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CVI. SOME PROPERTIES OF THE POLYSACCHARIDE COMPLEX EXTRACTED FROM A MARINE ALGA, *CHONDRUS CRISPUS*.

By MARGARET RUTH BUTLER.

From the Department of Biochemistry, University of Toronto.

(*Received January 22nd, 1934.*)

THE chemical nature of the water-soluble polysaccharide constituent of *Chondrus crispus* has previously received considerable attention. The interest manifested in it is attributable to its possession of marked gelatinising power. On account of this property it lends itself to a variety of uses. Unfortunately, the results to be found in the literature are based on variable methods of extraction, many of which are not very rational. The primary aim of the present work was, therefore, the preparation of a pure standard extract, so that the data obtained might be significant and of general application. The establishment of a definite criterion of purity is no simple matter, as the material is non-crystallisable and decomposes at very low temperatures. However, despite this, a reasonably pure standard extract has been obtained and from it have been prepared salts which give evidence of an even higher degree of purity.

HISTORICAL.

Investigation of this particular species, *Chondrus crispus*, and the material extracted from it in aqueous solution, dates from the work of Flückiger and Obermayer [1868]. In Abderhalden's *Biochemisches Handlexikon* [1911] there appeared the statement that "the pure mucilage of carrageen[1] corresponds to the formula $C_6H_{10}O_5$." The evidence on which this formula is based was not given. Though it is conceivable that it might represent the carbohydrate moiety, this formula is quite inadequate for the complete substance, since it takes no account of the 20 % ash content. There is also present some nitrogen which, until proven to be an impurity, cannot be disregarded. Apart from the fact that it is largely carbohydrate, very little is definitely known of the nature of the material in *Chondrus crispus* which is responsible for gelation. Haas [1921] advanced the theory that it is a colloidal electrolyte—an ethereal sulphate of calcium, the general structure of which might be represented by formula I.

$$R(OSO_2O)_2Ca \qquad\qquad Ca(OSO_2OR)_2$$
$$\text{I} \qquad\qquad \text{II}$$

Harwood [1923], making use of physico-chemical methods, finds the evidence contradictory and points out that without further experimental work it is impossible to choose between the alternative formulae, I and II. He appears, however, inclined to agree with Haas that I is more acceptable.

[1] The aqueous extract of *Chondrus* is commonly referred to in this way; the plant itself is also frequently called carrageen or Irish Moss.

On the other hand Haas's assumption of this disulphate ester has been criticised by Nelson and Cretcher [1931], on the grounds that the analytical data are insufficient to indicate this composition of the esters and to preclude the presence of polymerides of monosulphate esters.

Even should one of these ultimately be found correct, for the present one serves as well as the other as a working hypothesis. It is not impossible that both types may be present in the extract.

According to Haas, the calcium is ionised, but the sulphate ion is masked by its combination with the carbohydrate part of the molecule. On heating, this type of compound breaks down thus:

$$R(OSO_2O)_2Ca + 2H_2O = R(OH)_2 + H_2SO_4 + CaSO_4 \quad \text{.....(i).}$$

Later Haas altered his procedure and instead of using the dried extract with which he had previously worked [Haas and Hill, 1921] he prepared two fractions. One was a cold water extract (C.E.) and the other a hot water extract (H.E.) of the plants from which, presumably, all the C.E. had been removed.

The occurrence of a similar calcium sulphate-carbohydrate compound in agar-agar was reported in the same year [Neuberg and Ohle, 1921]. Haas and Russell-Wells [1923] made a fairly extensive study of a number of seaweeds. They found that in nearly all species having high ash contents the presence of an ethereal sulphate in the plants was indicated.

The evidence on the strength of which the ethereal sulphate formula was assigned is as follows:

(1) The calcium is freely ionised and can be quantitatively precipitated by ammonium oxalate even before hydrolysis.

(2) The sulphate content of the ash is high.

(3) The sulphate cannot be precipitated from the solution until after hydrolysis.

(4) The amount of the sulphate in the ash is only half of that in the hydrolysed solution. This loss of half the sulphate on ashing is attributed to its escape as sulphuric acid, according to equation (i).

Experiments with algae other than *Chondrus crispus* showed variations in the 2:1 ratio of "sulphate by hydrolysis" to "sulphate in ash." Nevertheless, Haas believes it permissible to assume, when the amount in the hydrolysed solution is greatly in excess of that in the ash, that the sulphate occurred in the plant in an ethereal linkage of the type described. The discrepancies from the 2:1 ratio are explained, in the case of higher ash sulphate, as due to the retention by an alkaline ash of some of the sulphuric acid which should normally have escaped. In cases where the sulphate in the ash is less than the theoretical, the loss is attributed to the presence of an ammonium salt, which would leave no ash.

Experimental.

Preparation and analysis of samples.

Preparation. From a number of methods of extraction and precipitation investigated, the following was finally adopted as being the most satisfactory method of obtaining a standard preparation.

20 g. of *Chondrus crispus* plants are washed with cold distilled water until free from chlorides. (Three or four washings are usually sufficient.) The washed *Chondrus* is then transferred to a flask with a litre of distilled water, and heated on a boiling water-bath for 5 or 6 hours. The resultant viscous mixture is centrifuged and the clear liquid filtered through a Chardin filter-paper on a Büchner funnel encased with a hot water jacket. It is necessary to change the filter-paper

frequently. The filtrate, kept in constant motion by stirring, is evaporated on a water-bath to approximately 300 ml. It is then poured, slowly and with stirring, into one litre of 95 % alcohol. A stringy, fibrous material is precipitated, which is removed as soon as formed, allowed to drain through cheese-cloth and finally transferred to 250 ml. of 95 % alcohol, in which it remains until the next day. It is then transferred daily from one to the next of the following washes: (1) and (2) 200 ml. absolute alcohol; (3) and (4) 150 ml. anhydrous ether. The precipitate is then dried in a vacuum-desiccator for a day and finally ground to a fine powder. It is dried in air for at least 6 hours at approximately 80° before being used. If the temperature is allowed to rise above this for any length of time charring occurs. The product is a creamy-white, non-crystalline powder, soluble in both cold and hot water and forming in 2 % solution a firm gel.

Ash. The ash content of the extract does not represent simply salt content but combined inorganic material. It does not, as will be seen later, represent all of this, but it may be expected to represent approximately the same percentage of the combined inorganic material in all cases, since the percentage lost depends on the original structure. Ash determinations of a number of extracts prepared from different collections are given in Table II.

It will be observed that the differences in ash content are slight, in spite of the fact that these extracts are made from plants collected at different places and in different seasons (Table I).

Table I. *Identification of samples.*

Sample	Place of collection	Month of collection
S	South Shore, P.E.I.	August
N	Souris, P.E.I.	,,
H	Halifax, N.S. (Pt. Pleasant)	February
T	,, (McNab's Island)	August
D	,, (Devil's Island)	June
P	Peggy's Cove, N.S.	August
C	Cavendish, P.E.I.	,,
F	Details of collection missing. Extract made without any washes of absolute alcohol	

The standard extract used in the present investigations was prepared from sample C.

Table II. *Percentage of ash in different extracts.*

Sample	% ash (dry weight)	Sample	% ash (dry weight)
F	17·30	S	19·11
D	19·32	N	18·43
T	19·46	H	16·36
P	19·74	C	19·44

As previously noted, many investigators regard the ash content of marine algae as practically pure calcium sulphate. The fact that the SO_4 content of the ash (see Table VI) averages only 58 % negatives this assumption. (SO_4 calculated for calcium sulphate is 70·34 %.)

The ash was found to contain varying quantities of potassium up to 12 % (Table VI). Russell-Wells [1922] reports that the ash of her extract contained sulphate, calcium, magnesium and small quantities of sodium and potassium, with traces of iron. The extract, therefore, probably contains a calcium ethereal sulphate, with which there are associated analogous salts of other metals. There is found also some phosphate which was not originally precipitable as the phosphate ion, and which may be assumed to have been present in an organic combination.

Sulphate. During the present investigation, sulphate (SO_4) was determined in three different preparations: (1) in hydrolysed samples; (2) in oxidised

samples; (3) in the ash. In each case the sulphate was precipitated by means of barium chloride, ignited and weighed.

Hydrolysis. In order to ascertain when hydrolysis was complete, a number of methods have been tried. The results (Table III) show that the sulphate is much more resistant to hydrolysis by alkali than by acid. It is evident also that all the sulphate is freed by boiling directly, for 5 hours, in 5 % hydrochloric acid, whereas even after 13 hours on the water-bath nearly 2 % still remains combined. This latter fact might be taken to indicate that the sulphur occurs in more than one type of combination.

Table III. *Sulphate by hydrolysis of extract.*

Method of hydrolysis	Time (hrs.)	% sulphate (SO_4)
Sample C:		
Boiled directly with 5 % hydrochloric acid	5	27·66
,, ,,	6	27·53
,, ,,	13	27·44
On water-bath with 5 % hydrochloric acid	6	21·62
,, ,,	13	25·62
Boiled directly with 5 % sodium hydroxide	6	13·32
Sample H:		
Boiled directly with 5 % hydrochloric acid	6	25·18
Boiled on sand-bath with 40 % hydrochloric acid*	6	25·38

* [Haas and Russell-Wells, 1923.]

Oxidation. The oxidation was accomplished by fusion with a hydroxide-nitrate (8:1) mixture. The melt was dissolved and acidified with hydrochloric acid, after which the sulphate was precipitated with barium chloride (Table IV).

Table IV. *Sulphur in extract after oxidation.*

Sample*	% sulphate (SO_4)	Sample*	% sulphate (SO_4)
I	29·04	W	26·70
	28·08		27·72
II	27·60	A	28·20
	29·07		29·28
III (*a*)	28·56	3X	30·24
	28·95		30·09
III (*b*)	28·02	5X	28·53
	27·96		
H	28·95		
	29·31		

* Differing symbols here indicate slight variations in the standard method of extraction, made with another purpose in view, but all are from the same collection of plants, namely sample C.

The sulphate after oxidation was found to average 28·38 %. The difference between this figure and that obtained by the hydrolysis, being very little outside the range of experimental error, seemed insufficient to warrant the assumption of any appreciable amount of unoxidised sulphur.

Ash. From the ash of the standard sample C, the sulphate was found to be 11·54 %, which figure does not satisfy equation (i), since in the present case only 35 % of the sulphate has been retained, rather than 50 %. Russell-Wells [1922] reports more than 50 % of the sulphate to be lost on ashing and attributes the excess loss to the presence in the original of some ammonium ethereal

sulphate, the sulphur of which would of course be completely lost on ashing. In the present case, however, the excess loss is too great to be explained in this manner, because the amount of nitrogen present would be sufficient to account for only 1 % of sulphate at the most (unpublished data).

As noted above, the ratio of "sulphate in hydrolysate" to "sulphate in ash" was found to be more nearly 3:1 than the 2:1 theoretically required by Haas's formula. This suggests the possibility that there might occur in the molecule three sulphate groups, only one of which is retained in the ash. An acid salt of the structure shown in the following equation would explain the observed results:

$$\underset{\text{III}}{HOSO_2O.R(OSO_2O)_2Ca} + 3H_2O = R(OH)_3 + 2H_2SO_4 + CaSO_4 \qquad \text{.....(ii).}$$

The sulphate retained in the ash of a compound such as III is one-third of that in the original.

Nelson and Cretcher [1931] found, in their analysis of the carbohydrate acid sulphate of *Macrocystis pyrifera*, that the percentage of ash obtained without oxidation was somewhat lower than when the sample was ashed in the presence of sulphuric acid. This they believe to be due, not to the retention of unoxidised sulphur in the latter case, but to the reduction of some sulphate in the former.

In order to determine whether or not the sulphate being lost here, in excess of that normally escaping as sulphuric acid, was due to this cause, approximately 0·2 g. of the sample was ashed in an electric muffle furnace, after having been moistened with nitric acid. A sample was ashed in the same manner with sodium carbonate (Table V). These figures show plainly that the sulphate loss

Table V. *Percentage of ash and sulphate in extract of sample C after different methods of ashing.*

Method	% ash (dry weight)	% SO_4 calc. to original extract
Simple incineration	19·44	11·54
Ashed with nitric acid	19·33	11·67
Ashed with sodium carbonate	—	28·49

experienced on ashing cannot be attributed to reduction of the sulphate. That the sulphate obtained after hydrolysis is the true sulphate value is shown by the agreement between it and that obtained after ashing with sodium carbonate, as well as that from the fusion with hydroxide-nitrate mixture (see Table III).

Preparation of salts.

Potassium salts. A 1 % solution of the extract was dialysed in collodion membranes[1] against frequently replaced potassium chloride until the dialysate gave a negative test for calcium (5 % potassium chloride was used at first, then 2·5 %). The dialysed material was then tested to make sure that no calcium had been retained. Dialysis was then continued against distilled water, until the excess of potassium chloride was removed. It was noticed that shortly after dialysis against the potassium chloride began, the extract became opaque and soon set to a gel. During continued dialysis against distilled water the material gradually liquefied.

[1] These membranes were made with the following collodion solution: 5 g. pyroxylin, 25 ml. absolute alcohol, 65 ml. anhydrous ether, 10 ml. ethylene glycol. Membranes were allowed to dry 25 to 30 minutes before being filled with water [Pierce, 1927].

The material, now free from calcium, is concentrated on the water-bath, precipitated and dehydrated according to the standard method. The product, a fine, pure white powder, is the potassium salt or salts resulting from replacement of the calcium and other cations of the water-soluble salts which are present *in situ* in the plant.

This material was found to have an actual ash content of 25·56 %. In appearance, the ash obtained from the original extract is frequently a dirty fawn colour and fused, while that of the potassium salts is snowy white and powdery. This ash contained K, 45·18; SO_4, 55·14 %. K_2SO_4 requires K, 44·89; SO_4, 55·11 %.

Now the plant *Chondrus* itself contains[1] 4·58 %, and the standard extract 2·22 % of potassium. On the other hand, the material obtained by dialysis against the potassium chloride solution contains only 0·069 % of calcium. Further evidence of the purity of this product is found in the absence from it of any trace of phosphorus. Positive tests for phosphorus were given by all other extracts, except that in the preparation of which the plants had been washed for 3 days in running water before the extract was made. When the period of dialysis was prolonged the resultant preparations showed that most of the cations of the extract were replaced by potassium during the first few hours, and that subsequent dialysis affected but slightly the total amount of potassium introduced.

Ammonium salts. Ammonium salts were prepared in a similar manner to the potassium salts, except that the final solution of the ammonium salts was not precipitated but was evaporated to dryness. Complete dialysis in this case required from 2 to 3 weeks, sometimes more. This ammonium salt gives less than 1 % of ash, whereas that which Russell-Wells [1922] prepared by another method yielded 5 % ash. The original sulphate in this material was found by hydrolysis to be 28·49 %.

Calcium salts. The potassium salt prepared as described above is obviously a more nearly pure material than the original extract. It was determined therefore to regenerate the calcium salt from it by dialysing the potassium derivative against calcium chloride (a 2·5 % solution was used) until no potassium could be detected in the dialysate. This point was reached in about 48 hours, after which the excess of calcium chloride was removed by dialysis against distilled water. The solution of the "regenerated" calcium salt was then evaporated, precipitated and dehydrated as in the standard preparation.

The material obtained differed in appearance from both the original and the potassium salt and decomposed much more readily even than the original.

The ash values of the original and the "regenerated" calcium salt did not differ greatly. The sulphate and calcium in the ash, however, were found to be very different. Figures are given in Table VI, which includes the potassium salt, for the sake of comparison, as well as the potassium figures for all three.

Table VI clearly shows to be erroneous the assumption that the original extract is a calcium salt. For one thing its ash contains more potassium than calcium. The difference between the calcium contents of the original extract and of the "regenerated" calcium salt is very great. The simultaneous decrease in potassium is not sufficient to account for this, nor is the increase which would occur by substitution of calcium in place of the hydrogen in formula III sufficient to account for the difference. The presence of still other cations is thus

[1] This figure is for a different collection of plants from that on which the results presented here were obtained.

Table VI. *Comparison of ash and sulphate in prepared salts with standard extract.*

	% ash	% SO_4 in ash	% SO_4 in original calc. from ash	% SO_4 in original by hydrolysis	% calcium in ash	% calcium in original salt calc. from ash	% potassium in ash	% potassium in original salt calc. from ash
Original extract	18·67	58·44	10·91	26·82	2·88	0·52	11·99	2·22
	19·14	58·74	11·54	27·51	3·68	0·68	—	—
Regenerated calcium salt	20·96	68·72	14·40	29·06	32·22	6·75*	0·76	0·16
Potassium salt	25·73	54·47	14·05	27·32	0·28	0·07	45·42	11·70
	25·26	55·14	13·94	28·00	—	—	—	—
Ammonium	0·60	—	—	28·49	—	—	—	—

* Calcium by hydrolysis in this sample was 6·56 %.

indicated. Further, the percentages of calcium and sulphate in the ash of the "regenerated" calcium salt approximate to those of pure calcium sulphate (Ca, 29·66; SO_4, 70·34 %), while they differ markedly from those of the original extract.

It will be noted from Table VI that in the potassium salt the theoretical 50 % of sulphate is lost on ashing. If the cations of a substance such as that represented in formula III were replaced by dialysis against potassium chloride, a neutral potassium salt (IV) would be obtained. This, on ashing, would undergo the following changes:

$$\underset{\text{IV}}{2R(OSO_2OK)_3} + 6H_2O = 3K_2SO_4 + 3H_2SO_4 + 2R(OH)_3 \qquad \text{.....(iii).}$$

It will be seen that here one-half the sulphate is converted into sulphuric acid and lost, as was actually found to be the case with the prepared potassium salt. This fact, together with the already noted absence of calcium and phosphate and the purity of the ash, leads to the conclusion that this preparation is a true ethereal sulphate. In the "regenerated" calcium salt also a 2:1 ratio of "sulphate from hydrolysis" to "sulphate from ash" is established. The original extract, being a mixture of sulphates, the ratio of "hydrolysis sulphate" to "ash sulphate" in it has not the same significance.

Further evidence that the extract of *Chondrus crispus* cannot rightly be regarded as a pure chemical compound was obtained from an examination of

Table VII. *Comparison of fractions with standard extract.*

Tests	C.E.	H.E.	Standard
50 % alcohol	No ppt.	Good ppt.	Good ppt.
Molisch	+	+	+
Biuret	–	–	–
Reduction	–	–	–
Reduction after hydrolysis	+	+	+
Potassium	+	– ?	+
Phosphate	+	–	+
Chloride	None visible	– ?	– ?
Calcium	?	+ + +	+ +
Sulphate	– ?	–	–
Sulphate after hydrolysis	+	+	+
Ash	27·43 %	17·24 %	19·47 %
	26·43	17·35	19·42
	27·49	16·80	
Nitrogen	0·32	0·16	0·24
	0·30	0·15	0·24

the fractions described by Haas [1921]. Extracts were made according to his method of separation and compared with one another and with the standard extract, as previously described. Instead of being simply evaporated to dryness, however, the fractions were precipitated and dehydrated in the same manner as the standard preparation.

Solutions of the two fractions differ greatly in appearance; the H.E. is almost water clear, while the C.E. has a yellowish colour and is very opaque, making it difficult to distinguish precipitates in it. In appearance, as in other properties, the standard is intermediate between the C.E. and H.E. which might be expected, as it is in effect a mixture of the two, containing little C.E. and much H.E. The marked difference in ash content of C.E. and H.E. might be attributed to salts adherent to the plant and taken into solution along with the polysaccharide complex. To obviate this possibility the method of fractionation was modified in the following way. A suspension of the finely ground plants was made, introduced into collodion membranes and dialysed against frequent changes of distilled water. The first dialysates were tested and found to contain chloride, sulphate, calcium and potassium ions. The absence of the chloride ion—because it is the most abundant in sea-water and is detectable longer in the dialysate—was taken to signify that all the salts had been removed. The dialysed suspension was filtered through Chardin filter-paper on a Büchner funnel. The filtrate, which from this point was treated according to the standard method of preparation, yielded the "dialysed cold extract" (D.C.E.) which was free from adherent salts. The residue was then extracted with water on a boiling water-bath for 5 hours. From the filtrate of this extract was obtained, by precipitation and dehydration as before, the "dialysed hot extract" (D.H.E.). It was now possible to make a comparison of the ash material actually combined in the two fractions with that combined in the standard extract.

Table VIII. *Comparison of ash and sulphate content of fractions with standard extract.*

Sample	% ash (dry weight)	% SO_4 in ash	% SO_4 in original salt calculated from ash	% SO_4 in original salt by hydrolysis
Standard	19·42	58·74	11·54	27·51
D.C.E.	21·47	59·36	12·75	28·79
D.H.E.	17·63	60·90	10·74	27·83
H.E.	17·13	63·34	10·85	27·03
C.E.	27·12	—	—	—

Assuming that the difference between the ash of the C.E. and D.C.E. is due to free salts, there still remains a 4 % difference between the ash contents of the dialysed fractions (D.C.E. and D.H.E.). The ash content of the standard is intermediate between the fractions, as a mixture of the two would be. The ash of the D.C.E. contains more phosphorus than that of the D.H.E., which, of course, would help to increase the percentage of ash in the former.

A consideration of the sulphate content of the fractions may mean little or nothing, as the differences recorded are very small.

The original sulphate obtained both by hydrolysis and by calculation from the ash sulphate is higher in the D.C.E. The percentage of sulphate in the ash of the D.C.E. might be depressed somewhat by the presence there of phosphate. The difference in the "hydrolysis sulphate" between the fractions becomes more pronounced when the D.C.E. is contrasted with the H.E. instead of the D.H.E., because the H.E. contains none of the C.E., while the D.H.E. contains some

of it. In respect to sulphate content, therefore, as well as ash, the standard preparation would appear to fill an intermediate position.

It should be noticed, from Table VIII, that the proportion of "hydrolysis SO_4" to "ash SO_4" coincides no better with the 2:1 ratio in the case of the fractions, than it does in the standard as here prepared.

Comparison with agar-agar.

Carrageen is frequently compared with agar-agar. Both are products of marine algae, and possess a common physical characteristic of importance, namely the power to gel in aqueous solution. As the method of preparation of most commercial agars does not eliminate nearly all the impurities, a solution of agar was subjected to the precipitation and dehydration procedure described, before being compared with the standard extract of *Chondrus*. Table IX affords a comparison of the purified and unpurified agar with the standard extract of *Chondrus*.

Table IX. *Comparison of agar with standard extract of* Chondrus.

Tests	Unpurified agar	Purified agar	Standard *Chondrus* extract
Appearance of aqueous solution	Yellowish opaque	Bluish-white opaque	Colourless clear
Molisch	+	+	+
Reduction	?	?	–
Reduction after hydrolysis	+	+	+
Biuret	–	–	–
Calcium	+	+	+ +
Potassium	–	–	+
Chloride	+	+ ?	–
Phosphate	–	–	+
Sulphate	+	?	–
Sulphate after hydrolysis	+	–	+
Ash	4·44 %	2·17 %	19·11 %
Nitrogen	2·51	0·07	0·24

Though it appears from this table that there is no sulphate in the purified agar, this is contrary to conclusions of the majority of investigators [Samec and Ssajevic, 1921; Fairbrother and Mastin, 1923], and as it is the result of a single observation no great importance is attached to it. Even the unpurified agar gives a sulphate test only after hydrolysis of a concentrated solution, or in the ash. However, the basic structure of agar cannot include an ethereal sulphate group, as it does in the case of the *Chondrus* extract. Even supposing the whole ash were composed of sulphates, it would be sufficient to account for the existence of only a small fraction of the original material as an ethereal sulphate similar to that present in the *Chondrus* extract, unless indeed this were an ethereal ammonium sulphate. That it could have been such is precluded by the small amount of nitrogen present in agar. We seem, therefore, to be led to the conclusion that though agar contains a small quantity of ethereal sulphates, they are not such essential constituents as in *Chondrus*. If, on the other hand, it is assumed that the structure of agar is based on the ethereal sulphate linkage, it follows from the difference in ash content that the carbohydrate group is ten times as complex as that in *Chondrus* extract.

Discussion.

The data here presented afford evidence that the material extracted by water from *Chondrus crispus* is not a single substance, but a mixture of several, neither the whole nature nor the number of which is known.

The fractionation suggested by Haas [1921] appears artificial, as neither of the fractions is any nearer being a pure chemical compound than is the standard extract. Russell-Wells [1922] says, "the similarity of oxidation products of both (fractions), points to both consisting of substances belonging to the same group of compounds, but there is not evidence enough to show whether the differences are due to different bodies, or mixtures in different proportions of the same substances. On the whole the former of these alternatives appears the most likely." From the findings here presented, however, it seems more rational to regard the fractions as mixtures of the same or very closely allied substances, in different proportions and to suppose that in addition to the common components, each fraction may contain some substance or substances which do not occur in the other. Thus the C.E. contains inorganic and simple organic materials, *e.g.* phosphates, from which the H.E. is free. It is quite probable that some of the more complex constituents of the H.E. are entirely lacking from the C.E.

The evidence for the purity of salts prepared from the standard extract, on the other hand, is clear cut. In the case of the potassium salt, for instance, there is: (1) an agreement between the percentages of potassium and sulphate in the ash with those of pure potassium sulphate; and (2) an agreement of the sulphate content of the salt and its ash with the theoretical 2:1 ratio (Table VI). Furthermore (3) the removal of the phosphate ion which was present in the standard extract suggests that other impurities also have been removed.

If the potassium preparation is not a single salt, it can only be a mixture of ethereal sulphates of potassium, which are very similar in structure and differ only in complexity. The variations, being such as not to interfere with the agreements noted, must be either in the nature or the complexity of the carbohydrate radical with which the potassium sulphate is united, or in the number of potassium sulphate groups united with the (same) carbohydrate radical.

It has been determined that the sulphate forms 28 % of the whole potassium salt. On the basis of formula V, $R(OSO_2OK)_2$, the molecular weight would be roughly 685, or that of the carbohydrate radical 415. Based on formula IV, $R(OSO_2OK)_3$, the molecular weight would be approximately 1030, or that of the carbohydrate fraction 625. The properties of the material, however, are such as to suggest that the molecular weight is even greater than this. Therefore, one of the following formulae probably represents its constitution more correctly:

$$[R(OSO_2OK)_2]_n \quad \text{VI} \qquad \text{or} \qquad [R(OSO_2OK)_3]_n \quad \text{VII}$$

Since the total ash of this preparation can be accounted for it should prove a very satisfactory material for use in investigating the carbohydrate fraction of the complex.

Summary.

A method which yields a standard and relatively pure substance is described for the preparation of the gelatinising constituent of *Chondrus crispus*. This material is largely carbohydrate in nature but has an ash content equivalent to approximately 20 % of its dry weight.

The extract previously described as an ethereal sulphate of calcium is now shown to be a mixture of sulphates, together with other substances, for example, phosphates.

The potassium salts of the ethereal sulphates which occur *in situ* have been prepared, and the preparation found to be superior in purity to the original extract.

The potassium of this preparation was then completely replaced by calcium and the differences between these "regenerated" calcium salts and the original extract determined. The results invalidate the assumption that the original is solely, or even chiefly, an ethereal sulphate of calcium.

The ammonium salts also were prepared and are compared with those of potassium and calcium and with the original extract.

It was found that more sulphate was lost on ashing the standard extract than corresponded to the theoretical loss, on the basis of the formula proposed by Haas [1921]. This leads to the suggestion of a modified formula—that of an acid salt—which more closely fits the facts. Such an acid salt would, by the method used in the preparation of the potassium, calcium and ammonium salts, be changed into a neutral salt. The sulphate loss on the ashing of these prepared salts corresponds with the theoretical.

The *Chondrus* extract is compared with a purified sample of agar-agar, to which it is similar in physical properties and origin.

The present work has been made possible by assistance from the National Research Council of Canada and the Alumni Federation of the University of Toronto, to which organisations my sincere thanks are due. I am happy to have this opportunity of expressing to Prof. H. Wasteneys my appreciation of the encouraging interest and helpful advice received in his department.

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CVII. ON THE PHYSIOLOGICAL ACTION OF ASCORBIC ACID AND SOME RELATED COMPOUNDS.

By VICTOR DEMOLE.

From the Pharmacological Laboratories of Messrs Hoffmann-La Roche and Co., Ltd., Basle.

(*Received March 24th, 1934.*)

The biochemical action of *l*-ascorbic acid has hitherto been studied principally on the scorbutic guinea-pig. Only recently Harris *et al.* [1933] investigated the excretion of ascorbic acid in the human subject and found that with a normal intake of vitamin C about 30 mg. of ascorbic acid can be determined by dichlorophenolindophenol titration in the daily output of urine. The excretion is temporarily increased when large doses of vitamin C (150 ml. lemon juice corresponding to about 150 mg. of ascorbic acid are given). It was also found by van Eekelen *et al.* [1933] that with a normal diet about 25 mg. of ascorbic acid are excreted daily in the urine.

In order to determine if, as in the case of vitamins A and D, symptoms of hypervitaminosis occur after the administration of large quantities of ascorbic acid, the present author has investigated the tolerance of various laboratory animals (cold and warm blooded). The vitamin was administered *per os*, subcutaneously, intraperitoneally and intravenously, using an aqueous solution of ascorbic acid which was neutralised with sodium hydroxide shortly before use. In the following table the maximum single doses given to the various animals are summarised.

		Dosage in g./kg.			
Animal	Weight g.	Oral	Subcutaneous	Intraperitoneal	Intravenous
Axolotl	15–30	—	5	5	—
Frog	30–40	—	5	5	—
Mouse	12–20	5	5	2	1
Rat	120	5	2·5	—	1
Guinea-pig	250	5	1	—	0·5
Rabbit	2500	2	1	1	1
Cat	2500	1	0·5	—	0·5
Dog	8000	0·5	0·2	—	0·2

The doses were well tolerated in every case and no symptoms of hypervitaminosis were observed. Only in the mouse after oral administration was a brief laxative effect observed, this being probably caused by the relatively large quantities of salt administered. The body temperature of dogs and rabbits was not influenced for 24 hours after the injection. The Ca and P of the blood were not altered.

Ascorbic acid was also very well tolerated when administered over a long period. Guinea-pigs maintained on a normal diet received orally, subcutaneously or intravenously for 6 days 0·4–2·5 g. ascorbic acid per kg. daily (total dose 2·4–12 g./kg.). Under similar conditions mice were given 0·5–1 g./kg. for 7 days (a total of 3·5–7 g./kg.). The animals remained completely normal during the experiment and for 14 days thereafter. They showed no difference in appetite,

increase of weight and general behaviour from controls receiving the same amounts of the biologically inactive galacturonic acid. Histological investigation of various organs (kidney, pancreas, liver, heart and lungs) showed no definite changes.

As the minimum curative and prophylactic dose of ascorbic acid for the guinea-pig is 0·5–1 mg. daily and in the above experiments 2·5 g./kg. daily were administered, it is evident that the animals tolerate 500–1000 times the therapeutic dose of ascorbic acid daily without ill effects; the average weight of the guinea-pig being taken as 250 g. Experiments with a still higher dosage seem unnecessary as such is unlikely to occur in practice.

The reducing capacity of the urine for Fehling's solution or iodine in potassium iodide was definitely increased after administration of ascorbic acid. After intravenous or subcutaneous injection of 1–2 g./kg. of ascorbic acid to mice or rabbits, or after oral administration of 3–5 g./kg. to guinea-pigs, the urine obtained an hour later gave with Fehling's solution a pronounced precipitate of cuprous oxide. After 2–4 hours the reducing capacity reached a maximum and subsequently decreased fairly rapidly. After 20–24 hours the excretion was practically ended.

Method.

A dog weighing 9·6 kg. received 1 g. ascorbic acid, corresponding to approximately 0·1 g./kg. The urine was removed by catheter at intervals and the reducing capacity of the various fractions determined iodimetrically. The first fraction of urine (11 ml.), taken after 1 hour, showed a reducing capacity corresponding to a total amount of 0·153 g. ascorbic acid. In the second fraction (27 ml.), taken 3 hours after the injection, the reducing capacity corresponded to 0·377 g. ascorbic acid. The third fraction (27 ml.), taken after 5 hours, contained only 0·11 g. of ascorbic acid. The fourth fraction (100 ml.), taken after 8 hours, contained a total of only 0·057 g. of ascorbic acid. The reducing capacity of later fractions of urine showed normal values. The total excretion of ascorbic acid estimated iodimetrically was 0·701 g., *i.e.* about 75 % of the amount administered. The highest concentration of ascorbic acid (1·4 %) occurred in the second fraction of urine (1–3 hours after the injection). When still larger amounts of ascorbic acid are injected subcutaneously the concentration of ascorbic acid in the urine is increased still further and the excretion is continued for over 20 hours. The observed increase in the reducing capacity of the urine is undoubtedly due to an excretion of ascorbic acid because scorbutic guinea-pigs are cured by a daily dose of 0·2–1 ml. of this urine in 4–8 days.

The ascorbic acid in the circulation also increases the reducing capacity of the blood. After intravenous or subcutaneous injection of 0·4–0·5 g./kg. ascorbic acid to rabbits the apparent values for blood-sugar correspond to 140–160 mg./100 ml., the increase being only temporary. After one or two hours the blood-sugar values have again reached the normal of 110–120 mg./100 ml.

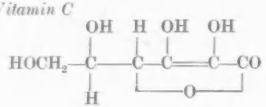
That this increased reducing capacity is not due to a mobilisation of glucose in the blood is proved by the fact that it is impossible to cure the convulsions of a hypoglycaemic rabbit by repeated injections of 0·1 g./kg. ascorbic acid intravenously or subcutaneously.

The action of related compounds.

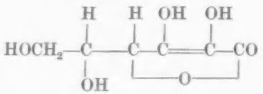
The researches of Reichstein *et al.* [1933, 1, 2; 1934] made it possible to investigate the antiscorbutic action of synthetic *l*-ascorbic acid and some of its isomerides and homologues. The complete identity in biological action of

synthetic *l*-ascorbic acid with the natural product from paprica was established, both when the synthesis was made from xylose and from sorbose as starting material. The isomerides and homologues investigated are shown below (in the case of the heptonic acids the lactone formulae are not given owing to the uncertainty as to whether a five- or six-ring is present):

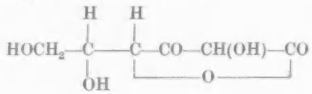
l-Ascorbic acid
Vitamin C



d-Ascorbic acid



d-Erythro-3-ketohexonic acid



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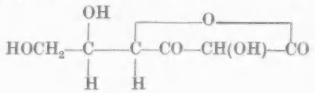
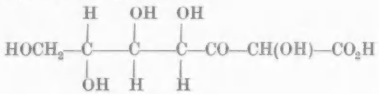
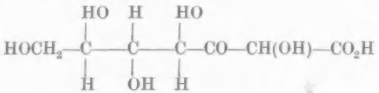


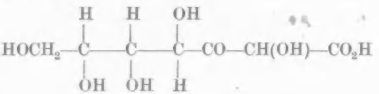
d-Lyxo-3-ketoheptonic acid



l-Xylo-3-ketoheptonic acid



d-Arabo-3-ketohexonic acid



The *d*-erythro-3-ketohexonic acid was found to have about 1/20 of the activity of *l*-ascorbic acid, in agreement with the result of Dalmer and Moll [1933]. The *d*-ascorbic acid was tested on the scorbutic guinea-pig and found to be completely inactive in doses 40 times as high (20 mg. daily) as were required for cure with *l*-ascorbic acid. The other homologues tested were also inactive in doses of 4–20 mg. daily.

Summary.

1. Large doses of *l*-ascorbic acid (up to 5 g./kg. in one dose or up to 12 g./kg. total amount in the chronic test) were tolerated without symptoms by our animals. Histological examination of the organs showed no change in the kidney, liver, heart and lung. There was no evidence of hypervitaminosis C.

2. After administration of 0·1 g./kg. ascorbic acid subcutaneously to a dog 75 % was excreted in the urine during the next 9 hours.

3. The urine of animals receiving large doses of ascorbic acid is capable of curing scorbutic guinea-pigs in daily doses of 0·2–1 ml. daily.

4. Synthetic *l*-ascorbic acid behaved biologically identically with *l*-ascorbic acid from paprica.

5. *d*-Ascorbic acid is inactive in doses of 20 mg. daily. Of the various isomerides and homologues examined only *d*-erythro-3-ketohexonic acid showed a definite antiscorbutic action which was about 1/20 of that of *l*-ascorbic acid.

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CVIII. THE EFFECT OF IODOACETIC ACID AND OTHER SUBSTANCES ON THE DISAPPEARANCE OF GLUCOSE FROM SHED BLOOD.

By DORIS MIDDLETON MOWAT and CORBET PAGE STEWART[1].

From the Clinical Laboratory, Royal Infirmary, Edinburgh.

(*Received March 31st, 1934.*)

Recent work on the breakdown of glucose to lactic acid, while it has produced a number of new facts and hypotheses, has not by any means succeeded in producing a clear and unequivocal account of the reactions involved. Thus on the one hand we have the demonstration that iodoacetic acid, which prevents lactic acid formation in muscle, reacts with and destroys glutathione the co-enzyme of glyoxalase. On the other hand, it has been claimed that muscle, from which gluthathione has been removed by dialysis, is still capable of lactic acid production provided that adenosinetriphosphate and magnesium ions are present.

The experiments described in this paper were undertaken in the hope that shed blood, in which the process of glycolysis is continuous, would prove a simpler system than muscle, the underlying assumption being that in both tissues lactic acid formation followed the same course. As will be seen, this assumption proved not to be justified, but this fact in itself seems to be one of considerable importance.

Experimental methods.

Human venous blood was used, with addition of sodium oxalate as an anticoagulant. In some cases the blood was diluted with an equal amount of water (a procedure shown to be without appreciable influence on the rate of disappearance of sugar) and in some the p_H was adjusted to 8·5 by addition of sodium hydroxide solution. This adjustment of p_H, while it increased the rate of glycolysis in the control samples, as compared with blood at the normal p_H, did not affect the inhibition of glycolysis by iodoacetic acid *etc.* Measured amounts of this blood mixture were transferred to separate test-tubes and to them the various substances to be studied were added in $N/10$ solution. Acids were used in the form of sodium salts (at p_H 7·5) and $N/10$ NaCl was used as a control, and, where necessary, for adjustment of volume. Incubation was at 37°. Sugar estimations were made by the method of Hagedorn and Jensen.

Iodoacetic acid was prepared by the action of potassium iodide on chloroacetic acid and purified by repeated precipitation from benzene solution with light petroleum. Iodoethyl alcohol was synthesised by the method of Hendry [1889], the final product being decolorised by sodium thiosulphate and distilled *in vacuo*. *l*-Bromopropionic acid was synthesised by the method of Volhard [1887] and from it a specimen of *l*-iodopropionic acid was prepared by the method of Abderhalden and Guggenheim [1908]. The β-iodopropionic acid was a Kahlbaum preparation, kindly presented to us by Dr H. S. Rule.

[1] In receipt of a part-time grant from the Medical Research Council.

Results and discussion.

Iodoacetic acid proved to be the most efficient of the substances tested in preventing the disappearance of glucose. Nevertheless it was without appreciable effect in concentrations of the order capable, in cardiac muscle, of completely inhibiting anaerobic carbohydrate breakdown. Bromoacetic acid was almost as active, chloroacetic acid less so; whilst iodoethyl alcohol and α-iodopropionic acid displayed smaller though very definite powers of inhibition of glycolysis. β-Iodopropionic acid however was quite inactive (Table I).

In a number of experiments it was noticeable that iodoacetic acid, while completely inhibiting glycolysis from the end of the first hour of incubation to the seventh hour, allowed some apparent decrease of sugar during the first hour. In part this may be due to the time necessary for the iodoacetic acid to exert its effect—*i.e.* to react with glutathione. Since, however, the method of protein precipitation used in the Hagedorn and Jensen method does not completely remove non-carbohydrate reducing substances, it seems likely that this apparent decrease in the blood-sugar may be due to the destruction by the iodoacetic acid of glutathione and possibly other substances such as ergothioneine.

The experiment quoted in Table I (*a*) shows the inhibition well maintained at 24 hours. This was not always the case, though usually the control, even then, showed the lowest sugar content. Most probably this was due to the fact that no special precautions were taken to exclude bacterial contamination.

Table I. *Effect of iodoacetic acid, iodoethyl alcohol, α-iodopropionic acid, bromoacetic acid and chloroacetic acid on the rate of disappearance of sugar from blood.*

(*a*) 0·7 ml. *N*/10 NaOH added to each 2·0 ml. blood. 0·25 ml. *N*/10 solution of reagent (neutral) or NaCl added per 5·0 ml. of blood mixture. Incubation at 37°.

mg. sugar per 100 ml. blood.

Time hours	Control	Iodoacetic acid	Iodoethyl alcohol	α-Iodopropionic acid
0	87	87	87	87
1	79	84	82	77
3	55	84	81	69
5	55	84	75	59
7	37	85	61	57
24	35	80	59	57

(*b*) As above, but 0·4 ml. *N*/10 chloroacetic acid, 0·2 ml. *N*/10 bromoacetic acid or *N*/10 iodoacetic acid (neutralised) and volumes adjusted with *N*/10 NaCl.

mg. sugar per 100 ml. blood.

Time hours	Control	Iodoacetic acid	Bromoacetic acid	Chloroacetic acid
0	225	225	225	225
3	201	224	215	209
5	179	209	211	91

Blood from a case of diabetes.

Chloro-, bromo- and iodo-acetic acids are all known [Dickens, 1933] to react with glutathione *in vitro*, and the velocity constants of the reactions have been determined. The action between glutathione and chloroacetic acid proceeds very much more slowly than those between glutathione and bromo- and iodo-acetic acid. In accordance with this, chloroacetic acid is much less efficient than the

other two substances in preventing glycolysis, but it is rather surprising to find it as efficient as it is—roughly half as efficient as bromoacetic acid. This is particularly so when one remembers that even in relatively greater concentrations it is without inhibitory action on the lactic acid production of cardiac muscle [Gaddie and Stewart, 1934]. These facts immediately suggest that the mechanism of sugar breakdown in blood differs from that in muscle and that the action of chloroacetic acid is not confined to the destruction of glutathione.

It is obviously desirable to test the effect of glutathione on the other two substances capable of inhibiting glycolysis (iodoethyl alcohol and α-iodopropionic acid) as well as the related compound, β-iodopropionic acid, which had no such effect. This became especially important when it was shown [Gaddie and Stewart, 1934] that none of these substances had an inhibitory effect on the frog's heart comparable with that exerted by iodo- and bromo-acetic acids. The method of testing was as follows: Solutions of the substance to be tested ($M/10$) and glutathione ($M/100$) were prepared at p_H 7·5, and 10 ml. of each solution were mixed with 10 ml. of a phosphate buffer at the same p_H. From this mixture, 5 ml. were at once removed and examined immediately for hydriodic acid. Further samples were removed at intervals of 15 minutes (the mixture meanwhile standing at room temperature) and tested in the same way. Control experiments were made without glutathione. Estimation of hydriodic acid was made by adding to the sample 2 ml. of $N/10$ potassium ferricyanide containing zinc sulphate, and 2 ml. of 3 % acetic acid, extracting the liberated iodine with chloroform (three extractions using 2 ml. of chloroform each time) and titrating the chloroform extracts with $N/200$ sodium thiosulphate. This method was tested on standard solutions of potassium iodide and was found to give satisfactory results.

When β-iodopropionic acid was tested in this way, the reason of its inactivity in preventing glycolysis became apparent at once—even in the absence of glutathione it lost hydriodic acid with much greater rapidity than did iodoacetic acid in presence of glutathione.

The α-iodopropionic acid, however, was stable alone and reacted with glutathione much more slowly than did iodoacetic acid (Table II), a finding in keeping with its smaller power of preventing the disappearance of sugar.

Table II. *Effect of iodoacetic acid, iodoethyl alcohol and α-iodopropionic acid on glutathione* in vitro.

ml. $N/100$ thiosulphate used in titrating iodine liberated per 5 ml. of solution.

Time min.	Iodoacetic acid	Iodoethyl alcohol	α-Iodopropionic acid
0	0·540	0·03	0·170
20	1·055	0·02	0·425
40	1·100	0·02	0·575
60	1·150	0·025	0·660

Iodoethyl alcohol, on the other hand, failed to react with glutathione, or rather, failed to liberate hydriodic acid in the presence of glutathione, although it was perfectly capable of inhibiting glucose breakdown. This can only mean that iodoethyl alcohol reacts with glutathione in some other way, or that its effect is exercised entirely on some other part of the system responsible for the conversion of glucose into lactic acid. The first of these alternatives seems to be excluded by the finding that the sulphydryl group (probably the essential part of the molecule for co-enzyme activity, and the most likely part to undergo

change) is unaffected by incubation with iodoethyl alcohol at p_H 7·5. This is shown by the fact that after incubation of the mixture for 1½ hours the amount of sulphydryl present is undiminished when tested both colorimetrically with sodium nitroprusside and titrimetrically with iodine. Still further evidence of the independence of glutathione and iodoethyl alcohol is afforded by the fact that added glutathione does not abolish the inhibitory action of iodoethyl alcohol on glycolysis, whereas it does abolish the action of iodoacetic acid (Table III).

Table III. *Effect of added glutathione on the inhibition of glycolysis in presence of iodoacetic acid and iodoethyl alcohol. Experiment as in Table I but 0·5 ml. reagent per 5 ml. blood mixture, and 20 mg. glutathione (in neutral solution) volumes adjusted with N/10 NaCl.*

mg. sugar per 100 ml. blood.

Time hours	Control	Iodoacetic acid	Iodoacetic acid and glutathione	Iodoethyl alcohol	Iodoethyl alcohol and glutathione
0	103	103	105	104	103
1	91	93	95	90	91
3	55	93	56	83	85
5	49	91	54	75	74

We are therefore driven to the conclusion that the actions of iodoacetic acid and iodoethyl alcohol, though leading to the same end-result, are fundamentally different at least in part. Moreover, since iodoethyl alcohol inhibits glycolysis in blood with an activity roughly half that of iodoacetic acid, but is totally ineffective in cardiac muscle at a concentration tenfold that of iodoacetic acid capable of complete inhibition of glycolysis, it follows that some part of the glycolytic mechanism present and of importance in blood is absent from cardiac muscle, or at least is not of critical importance in the latter.

The only alternative to this hypothesis appears to be the idea that iodoethyl alcohol is acting merely as a general enzyme poison. Indeed, when one considers the quantity involved, such an idea might be entertained with regard not only to iodoethyl alcohol, but also with regard to iodoacetic acid itself. For there is no proof that the whole action of this latter substance consists in its destruction of the co-enzyme of glyoxalase, and one might consider the restorative effect of added glutathione (which seems to be complete) as being merely an *in vitro* reaction resulting in the destruction of the enzyme poison and the replacement of the co-enzyme previously lost.

We have not tested this alternative hypothesis completely, and, indeed, there are not many enzyme systems in blood which have been thoroughly studied. We have, however, tested the effect of both iodoacetic acid and iodoethyl alcohol on the blood-phosphatase. The blood, made alkaline to p_H about 8·5 by means of sodium hydroxide was incubated with excess of sodium glycerophosphate solution and addition of sodium iodoacetate or iodoethyl alcohol in the concentrations known to inhibit glycolysis. In both cases samples withdrawn at 24 and 48 hours showed decreased hydrolysis of the organic phosphate as compared with the controls (Table IV). Since however the phosphatase may form part of the system responsible for the breakdown of glucose to lactic acid, this cannot be taken as evidence that the two iodo-compounds act as general enzyme poisons. It is perhaps noteworthy that they are without any inhibitory effect on the xanthine oxidase of milk.

Table IV. *Effect of iodoacetic acid and iodoethyl alcohol on blood-phosphatase.*

mg. P per 100 ml. blood.

Time (hours)	0	24	48
Control	7·44	27·18	39·17
Iodoacetic acid	7·18	21·44	31·63
Control	6·40	38·30	47·11
Iodoethyl alcohol	6·23	28·30	30·45

Summary.

Iodoacetic acid inhibits the disappearance of glucose from shed blood, but only in concentrations much higher than those required to prevent lactic acid formation in cardiac muscle. Bromoacetic acid has an almost equal effect. Chloroacetic acid, α-iodopropionic acid and iodoethyl alcohol have similar, though weaker actions. β-Iodopropionic acid is without effect probably on account of the rapidity with which it loses iodine.

The effect of iodoacetic acid, but not that of iodoethyl alcohol, is counteracted by addition of glutathione. Iodoethyl alcohol is also peculiar in not reacting with glutathione *in vitro* to produce hydriodic acid. Both iodoacetic acid and iodoethyl alcohol, in concentrations capable of preventing sugar disappearance, inhibit the action of blood-phosphatase.

The significance of these findings is discussed in relation to the mechanism of carbohydrate breakdown.

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CIX. A NOTE ON THE GENERAL INTERPRETATION OF FATTY ACID ANALYSES BY THE ESTER FRACTIONATION METHOD.

By THOMAS PERCY HILDITCH.

From the Department of Industrial Chemistry, University of Liverpool.

(*Received April 12th, 1934.*)

Misconceptions apparently persist, in the minds of some chemists, as to the degree of exactitude obtainable in, or claimed for, the analytical procedure which we, in common with a number of other investigators, have now employed for some years. It is desired, therefore, to make a brief general statement on the matter. Let it be clearly pointed out in the first place, that there are at least two essentially different general problems with which the student of the mixtures of fatty acids combined in natural fats is faced:

(*a*) Knowledge of the relative proportions, as well as of the individual acids, of what may be termed the *major component acids* of different natural fats. A "major component acid" is defined, broadly speaking, as one which occurs in quantities of from, say, 5–10 % upwards of the mixed fatty acids of a fat (and which very often amounts to 30, 40, 50 % or even more, of the total acids);

(*b*) Isolation and characterisation of acids which are sometimes present in much smaller proportions, and which form, for example, less than 1 % of the whole of the mixed fatty acids.

The essential difficulty of quantitative analysis of natural fatty acid mixtures resides in the presence therein of nearly consecutive members, saturated and unsaturated, of a single homologous series of compounds—acids, that is, of the C_{12}, C_{14}, C_{16}, C_{18}, C_{20}, C_{22}, *etc.* series. At the same time, natural fatty acid mixtures differ widely in complexity; for example:

	Major component fatty acids	
	Saturated	Unsaturated
Seed-fats of the cacao butter, *etc.*, type	C_{16}, C_{18}	C_{18}
Palm oil, tallow, lard, many vegetable oils	(C_{14}), C_{16}, C_{18}	C_{18}
Seed-fats of the *Palmae*	C_8, C_{10}, C_{12}, C_{14}, C_{16}	C_{18}
Milk-fats	C_4, C_6, (C_8, C_{10}, C_{12}), C_{14}, C_{16}, C_{18}	C_{18}
Marine animal fats	(C_{14}), C_{16}, (C_{18})	(C_{14}), C_{16}, C_{18}, C_{20}, C_{22}, (C_{24})

The ester-fractionation procedure is not suitable as a rule for quantitative determination of minute proportions of fatty acids (class (*b*) above), since an accuracy to within fractions of a unit % (of the total mixed fatty acids) cannot in general be reached by the method. On the other hand, for studies of the relative proportions of the major component acids of natural fats (class (*a*) above)—an aspect of fat-chemistry with which the writer and his colleagues have been engaged for a somewhat long period—the method gives results which,

at least with many of the simpler kinds of fats illustrated above, are reliable to about one unit %. Such an order of accuracy, or even somewhat less, is quite sufficient to have demonstrated, for example, many cases of empirical but close relationship between fatty acid compositions and biological origin; or, again, in one category of animal depot fats, to have established the relative constancy of palmitic acid content and the approximate stearic-oleic acid balance, which we have shown to be characteristic for fats of varying average unsaturation in this group.

Component fatty acids are given, therefore, to the first decimal place (in conformity with the usual convention that one more significant figure is given than that upon which reliance may be placed); also, the data are expressed as the percentage proportions of the *mixed fatty acids* estimated (so that the circumstance that the totals amount to exactly 100·0 in all cases has no bearing upon the experimental accuracy or otherwise of the determination). Moreover, it sometimes happens that the calculation ultimately results in the appearance of a fractional percentage (*e.g.* 0·7 % myristic acid, Table I, p. 781) of an acid present in small amount; in such cases of course this does not imply that narrower limits of accuracy are claimed for the analytical procedure employed than those already stated. These explanatory remarks will probably be superfluous to most workers in this field, but apparently the points referred to have not always been appreciated by some of our fellow-investigators.

When the ester-fractionation process is examined critically, it is at once evident that its order of accuracy depends almost wholly on the answer to the question: Is it possible to obtain ultimate ester-fractions in any one of which no more than two adjacent homologous saturated, and two adjacent homologous unsaturated, members are present? In all probability, absolutely complete separation in this respect is rarely, if ever, attainable; but due observance of two precautions adequately safeguards the analysis and leads ultimately to the presence of not more than traces of a third homologue in the fractions finally obtained:

(*a*) In the first place, separation by fractional distillation of esters is never attempted before having recourse to as thorough a separation as possible of saturated from unsaturated acids by the lead salt-alcohol process. This removes all stearic and higher saturated acids and very nearly all palmitic acid from the unsaturated acids. In the common cases therefore where mixtures of palmitic, stearic, oleic and linoleic acids (with perhaps minor amounts of myristic or other acids) are concerned, this simplifies very much the character of the two groups of esters which have to be distilled. In other and more complex instances, such as animal milk fats, which contain a good deal of saturated acids of lower molecular weight than palmitic, or the marine animal oils, with a mixture of homologous unsaturated acids, the lead salt separation is even more vital a part of the process; for here, even when it does not result in an almost complete division as between saturated and unsaturated acids, it still has the effect of removing almost all the palmitic and all higher saturated acids from the so-called "liquid" acids, with the consequence that there is a much greater differentiation in the relative molecular weights (and therefore boiling-points of the esters) of the saturated and unsaturated acids present in the "liquid" esters than exists in the original mixed acids as a whole, in which the C_{16} and higher saturated acids are also present.

(*b*) In the second place, in addition to such refractionation of primary fractions as may be necessary, it is our practice to collect a large number of fairly small final ester fractions, whether the composition of the mixed esters is

relatively simple or not. This multiplication of fractions minimises any error due to the possible retention of traces of a third homologue in fractions consisting almost wholly of two adjacent homologues, and also tends to reduce the effect of small positive and negative errors in the numerous equivalent and iodine value determinations involved.

Comparison of the closely similar compositions deduced by use of this method, in this laboratory and elsewhere, for the acids in different specimens of the same kind of fat is sufficient evidence of the fact that at all events it leads to results which are very concordant; but it may be argued that this does not necessarily imply their accuracy. On the other hand, the mixture of acids from a completely or partially hydrogenated fatty oil is of an essentially different type, so far as the analysis is concerned, from that from the original fatty oil, since the proportion of saturated acids is entirely altered by hydrogenation. A comparison of the results obtained from analyses of original and hydrogenated fats thus affords a somewhat rigorous test of the general accuracy of the procedure. A few instances of the kind, drawn from data in our laboratory records, will therefore be given by way of illustration; it is of course necessary to compare the molar percentages of the various homologous acids present in each case.

Table I.

Palm oil fatty acids (mol. %).

	Original		After hydrogenation	
Myristic	1·9		0·7	
Palmitic	34·3		35·5	
Stearic	5·3		62·7	
Oleic	50·6	63·8	1·1	63·8
Linoleic	7·9		—	

Pig back fat fatty acids (mol. %).

	Original		Partially-hydrogenated (1)		Partially-hydrogenated (2)	
Myristic	3·5		2·8		2·2	
Palmitic	26·6		25·6		28·1	
Stearic	14·0		18·1		28·2	
Oleic	41·4	69·0	50·9	69·9	40·5	68·7
Linoleic	13·6		0·9		—	
C_{20-22} unsaturated	0·9		1·7		1·0	

Elasmobranch liver oil fatty acids (mol. %).

		Original		After hydrogenation
C_{14}	Saturated	1·4	1·6	2·5
	Unsaturated	0·2		—
C_{16}	Saturated	15·3	19·4	17·5
	Unsaturated	4·1		—
C_{18}	Saturated	1·4	38·6	39·8
	Unsaturated	37·2		—
C_{20}	Saturated	1·1	16·8	16·8
	Unsaturated	15·7		—
C_{22}	Saturated	—	13·9	13·4
	Unsaturated	13·9		—
C_{24}	Saturated	—	9·7	10·0
	Unsaturated	9·7		—

It seems therefore that an accuracy of about one unit % can be relied upon for the fatty acids of the simpler fats while, as suggested by Guha *et al.* [1930], in

the more complex fats, especially in the marine animal oils, the figures may be less reliable and the order of accuracy perhaps only within 3–4 units %. Even the latter represents a not unreasonable standard of accuracy, compared with that attainable in the quantitative assay of many other natural products of a complexity comparable with that of most fat mixtures. Moreover, it has sufficed to demonstrate general relationships amongst the natural fats of the kinds to which reference has already been made; while the detection of palmitoleic acid in bird and rat fats (discussed in recent papers) is entirely due to the use of the ester-fractionation method.

Some recent work on the linoleic acid content of butter-fat by Bosworth and Brown [1933] and by Eckstein [1933] will now be considered. Bosworth and Brown succeeded, as a result of repeated fractionation of the mixed esters from over 11 kg. of butter-fat, in isolating and defining the minute proportions of decenoic and tetradecenoic acids which (as Grün *et al.* [1922; 1924] had previously shown) occur in butter. This outstanding contribution to knowledge of the minor component fatty acids of butter suggests that decenoic acid may form 0·18 % of the total fatty acids and, since the authors actually isolated an amount of tetradecenoic acid smaller than that of decenoic acid, it seems fair to assume that tetradecenoic acid occurs in still smaller quantities. (Bosworth and Brown consider however that the original butter must have contained 0·87 % of tetradecenoic acid.) They also show that highly unsaturated acids (probably of the C_{22} series) are present in butter-fat: and in an earlier paper [Brown and Sutton, 1931] the presence of such acids to the extent of 0·3 % was demonstrated.

In the course of their work however the authors state that they have been unable to demonstrate the presence of ordinary linoleic acid and suggest that the unsaturation higher than that of oleic ester, which invariably has been observed in our "liquid" ester fractions corresponding in equivalent to those of C_{18} unsaturated acids, is due to imperfect fractionation and contamination of these fractions by either the mono-ethenoid C_{10} and C_{14} or the polyethenoid C_{22} esters, or both.

Unfortunately, Bosworth and Brown, although they refer to our use of the methyl ester distillation method, have based their fractionation practice on the earlier work of Crowther and Hynd [1917], Holland and Buckley [1918–23], and Channon *et al.* [1924]. They prepared their esters by direct "methanolysis" of butter-fat and employed no preliminary resolution of the mixed acids into predominantly saturated and predominantly unsaturated groups. Moreover, they refer to criticisms advanced by Channon *et al.* against the ester-fractionation method, but overlook the very complete refutation of these criticisms which followed almost immediately from Armstrong *et al.* [1925]—a refutation which, so far as the present author is aware, has been accepted. (For example, recently, Drummond and Gregory [1932] refer to certain analyses as having been made "by the more accurate fractionation method.")

The iodine values of the acids in the higher methyl ester fractions obtained by Bosworth and Brown do not, it is true, suggest the presence of C_{18} unsaturated acids less saturated than oleic. This is natural, for, owing to the absence of any lead salt separation, methyl stearate and also (judging from the recorded mean molecular weights) fairly large amounts of methyl palmitate are present in all the fractions concerned. Since, in our experience, the minimum amount of stearic acid which has been observed in any butter-fat is greater than the maximum amount of linoleic acid which we have ever suggested to be present, it follows that even if complete separation of palmitic and C_{18} esters had been

achieved, the iodine value of their C_{18} esters would still have been somewhat below that of methyl oleate. Bosworth and Brown however applied a lead salt-ether separation to the acids from their higher ester fractions and supplemented this by attempted resolution of the unsaturated acids by means of the barium salts in benzene, and they conclude that practically all polyethenoid acids present are of higher molecular weight than the C_{18} series. The absence of data as regards the relative weights of the various fractions obtained in this part of their work limits any discussion to a qualitative basis, but it must be pointed out that the mean molecular weights of the acids from the insoluble (as well as from the soluble) barium salts are, in six instances out of nine, considerably higher than those of the original mixtures.

Bosworth and Brown, from an examination of the ether-insoluble bromo-addition products, were unable to demonstrate the presence of ordinary linoleic acid, or of more highly unsaturated acids of the C_{18} series. Eckstein [1933], employing the same method, finds evidence of only traces (of the order of 0·1–0·3 %) of linoleic and linolenic acids. The latter author also shows that addition of 5 % of the esters of either of these acids (prepared by the method of Rollett [1909]) to butter results in the mixed fat showing bromine values corresponding to 50 % or less of the added linoleic or linolenic acid: he obtained however by fractionation of the "liquid" methyl esters of a Detroit butter iodine values of 93, 98 and 117 for the three highest-boiling fractions.

Now, in all our analyses of butter-fat "liquid" methyl esters, there have been obtained consistently large fractions of esters with equivalents approximating to those of the C_{18} group, the iodine values of which range from about 92 to 99, that is, from 8–15 % above that of methyl oleate. In each of our papers on butter-fats [1929; 1930; 1933] only one instance was given, in each case, of the full details of the fractionation data, in an endeavour to save space. Seven other instances, taken from the material dealt with in these previous papers, will now be cited in order to emphasise the invariable nature of these observations. In doing so (again for considerations of space) only the weights, saponification equivalents and iodine values of the three or four highest-boiling fractions of the "liquid" esters will be quoted in each case, together with the total weights of "liquid" esters distilled and the proportion of the total fatty acids represented by the "liquid" fatty acids; the latter data are very material to the present argument.

Inspection of the figures (Table II) shows that the ester fractions of iodine value 92–99 to which attention is invited represent from 15 to over 20 % of the total fatty acids of the butter-fats. Bosworth and Brown [1933] say "This high iodine number can easily be explained now by the presence of low molecular weight acids with one double bond and iodine numbers greater than that of oleic acid together with appreciable amounts of unsaturated acids of high molecular weight with two or more double bonds." This statement is untenable for the following reasons. The iodine value of decenoic acid is 149·3 and of tetradecenoic acid 107·7. It would therefore require a mixture of about 50 % each of methyl tetradecenoate and oleate, or of about 20 % methyl decenoate with 80 % methyl oleate to give iodine values in the neighbourhood of 95; the percentage of a tetra-ethenoid C_{22} ester (iodine value 294) required to raise the iodine value of methyl oleate and the latter to a mean value of 95–97 would be less, namely, about 5 %. Consequently, the amount of any or all of these esters necessary to be present in our C_{18} ester fractions in order to produce the observed results would have been many times greater than the total amounts of these acids present in the whole of the butter-fat, according to Bosworth and Brown.

Table II.

Publication	Butter-fat No.	Proportion of "liquid" acids from total butter-acids (%)	Weight of "liquid" esters distilled g.	"Liquid" methyl ester fractions			
				No.	g.	Sap. equiv.	I.V.
Hilditch and Jones [1929]	"B"	30·7	91·41	L 3	19·07	290·1	91·0
				L 4	8·47	293·0	94·9
				L 5	26·29	293·6	99·3
				L 6*	7·17	325·2	103·0
Hilditch and Sleightholme [1930]	"I"	47·5	69·43	L 4	9·55	292·9	92·1
				L 5	7·33	294·5	93·5
				L 6	7·83	295·8	94·1
				L 7*	8·45	307·1	105·0
"	"V"	41·8	105·15	L 4	13·10	290·1	91·7
				L 5	9·27	293·5	95·1
				L 6	15·59	295·2	98·9
				L 7*	7·12	327·7	118·6
Bhattacharya and Hilditch [1931]	Cow ghee no. 2	44·2	48·70	L 4	8·63	293·8	92·4
				L 5	9·15	294·4	96·8
				L 6*	5·32	303·8	103·3
Dean and Hilditch [1933]	"Winter ration"	48·5	120·24	L 4	16·46	290·3	92·8
				L 5	20·75	293·5	94·0
				L 6	10·47	296·1	96·2
				L 7*	7·40	324·0	95·3
"	"Intermediate period"	52·0	120·31	L 3	14·35	291·4	90·8
				L 4	15·64	293·1	94·4
				L 5	16·52	294·2	96·3
				L 6	10·93	294·8	97·9
				L 7*	15·97	308·1	101·5
"	"Summer pasture"	52·2	120·61	L 4	15·63	292·4	91·6
				L 5	14·17	294·2	93·4
				L 6	20·25	295·0	96·4
				L 7*	15·31	308·3	99·3

* The final fractions, in each case, are the residual esters which remained undistilled; the iodine values of these are always notably higher than those of the preceding distillate, and doubtless this is due to the small traces of C_{22} unsaturated acids observed by Brown *et al.*, as well as to unsaponifiable matter (*e.g.* the residual esters, I.V. 118·6, from fat "V" above, still possessed an I.V. of 103·6 and an equivalent of 303 after removal of unsaponifiable matter). We have never attempted to allow, in the butter-fat analyses, for anything but C_{18} unsaturated esters and unsaponifiable in the residual fractions, because the iodine values thereof are probably of doubtful accuracy owing to the possibility of some thermal decomposition; we have therefore applied, for purposes of calculation, the iodine value of the preceding fraction to the C_{18} esters in the residues.

(In the instance referred to, allowance for C_{22} esters would lead to the fraction L 7 being recorded as 5·80 g. C_{18} and 0·78 g. C_{22} esters with 0·54 g. unsaponifiable, whereas our calculation (with C_{18} esters alone) was 6·43 g. C_{18} esters and 0·69 g. unsaponifiable. On the whole fat, this would lead to about 0·2 % diminution in the recorded C_{18} unsaturated acids and correspondingly to 0·2 % of C_{22} acids, calculated on this basis.)

The writer remains convinced, then, that these characteristic iodine values in the neighbourhood of 95 are due to unsaturated C_{18} esters. Bosworth and Brown state that there is no tetra-ethenoid C_{18} ester present, and Eckstein fails to find more than about 0·3 % at most of linoleic and linolenic esters in the butter-fat as a whole. Hilditch and Jones [1929] also state that they failed to isolate either any tetrahydroxystearic acid or any definite brominated acid (other than a trace insoluble in ether which darkened without definitely melting at 170–180°) from the ester fractions of iodine value 97–98. The consistent presence of C_{18} ester fractions corresponding to 15–20 % of the total butter-fatty acids with an average unsaturation more than 10 % in excess of that of methyl oleate nevertheless still requires explanation.

It is hoped to return to the investigation of this question in due course, but it will necessarily be some considerable time before any further report can be made on such experiments, and for this reason it has been thought best to point out the above facts in the present communication. It need only be added that Eckstein's experiments with regenerated linoleic and linolenic acids (presumably originating from seed fats) merely indicate that the ordinary linoleic acid of seed fats is not present to any marked extent in butter, and that Smith and Chibnall [1932] had similar difficulty in correlating the brominated products from the glyceride fatty acids of forage grasses with those from the ordinary linoleic acid of seed fats. Possibly this may afford a clue to the solution of an admittedly puzzling problem. In the meantime, the quantitative data, to which attention has here been redirected, must be taken into consideration in computing the general composition of butter-fatty acids, and the writer has adopted the view, from the 1929 paper onwards, that, in the absence of definite characterisation, the general distribution of C_{18} unsaturated acids in nature favours the assumption that the most likely polyethenoid C_{18} unsaturated acid to be present is diethenoid rather than still less saturated in character.

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CX. THE BODY FATS OF THE HEN.

By THOMAS PERCY HILDITCH, EDWIN CHARLES JONES and ARTHUR JAMES RHEAD.

From the Department of Industrial Chemistry, University of Liverpool.

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The examination of bird body fats has hitherto been restricted to separation of the saturated acids present by means of their lead salts (Twitchell) or by oxidation (Bertram). Bömer and Merten [1922] state that the acids present in a sample of goose fat were palmitic (21·8 %), stearic (3·9 %) and oleic (74·3 %), while Grossfeld [1930; 1931] reports for the same fat lower volatile acids (0·2 %), palmitic (20·9 %), stearic (10·6 %), oleic (49·0 %) and linoleic (19·3 %) acids[1]. The latter author also gives two similar component fatty acid analyses for chicken fat as follows: lower volatile acids (0·1 %), palmitic (18·4, 19·3 %), stearic (8·9, 7·5 %), oleic (54·7, 55·4 %) and linoleic (17·9, 17·8 %) acids. Grossfeld pointed out however that the percentage of palmitic acid, calculated from the saponification value of the total mixed fatty acids, was about 10 units higher than that calculated from the molecular weight of the separated "solid" fatty acids and attributed this to the presence of fatty acids of lower molecular weight than that of palmitic acid.

The work about to be recorded proves that this discrepancy is not due in hen body fats to the presence of lower saturated fatty acids, but to that of about 7–8 % of a palmitoleic acid. Indications of this or a similar acid were recently reported by Banks *et al.* [1933] in rat body fats, although lack of material prevented decisive evidence being obtained.

The primary objectives of our experiments were to ascertain the general nature of a bird fat and to compare the depot fats from different parts of the body. We were fortunate in having the co-operation of Dr E. M. Cruickshank, who not only supervised the rearing of the fowls, but was also good enough to separate for us the fatty tissues from the abdominal layers, gizzards[2] and necks of the birds. We wish to express our great appreciation of the help thus afforded to us by Mr Halnan and Dr Cruickshank of the Cambridge School of Agriculture.

The specimens of the three body fats which we examined in the first instance were from 12 Light Sussex hens, 2 years old, whose diet had included 7 % of fish meal containing about 2·5 % of fish fat. The results of analyses by the ester fractionation method showed, as stated, that palmitoleic acid and also small amounts of unsaturated acids of the C_{20} and C_{22} series were present; and a new series of fats from 24 Light Sussex hens (which had received no fish meal), 7 months old, was therefore studied, in order to ascertain whether these acids

[1] The figures given here are the percentages of each acid on the *mixed component fatty acids*, and not (as in the original memoirs) those in the whole fats.

[2] The "gizzard fat" consisted of the layer of fat adherent to the gizzard together with the fat of the mesenteric membrane and any which was packed around the kidneys. Roughly speaking therefore this fat might perhaps be compared with the leaf and inner back fats of the pig, whilst the abdominal layer is in some respects more akin to the outer layers of back fat of the pig.

originated from those present in the fish meal fat. The results however were closely similar to those for the original group, and it is inferred that these acids are normal components of hen depot fats.

Since the diet of the second batch of 24 hens did not contain fish meal, it is perhaps preferable to deal with them first in the present paper. The diet of each group of birds, from 6 weeks after hatching onwards[1], was made up of mash (bran, maize and Sussex ground oats 20 parts each and sharps 40 parts) and grain (maize and wheat). For the group of 24 hens, 10 % of extracted soya bean meal and 2 % of mineral mixture were added to the mash, whilst to the mash given to the group of 12 hens there were added 7 % each of extracted soya bean meal and fish meal (containing 2·4 % fat) and 3 % of mineral mixture.

The average analyses of the rations were as follows:

	Moisture %	Ether extract %	Protein %	Carbo-hydrate %	Fibre %	Ash %
24 hens (fish meal-free)	12·4	4·0	16·1	55·3	6·5	5·7
12 hens (with fish meal)	12·5	3·8	14·1	57·9	6·6	5·1

The fats were extracted from the tissues by boiling with acetone; at the ordinary temperature they consisted chiefly of deep-yellow liquids from which a certain quantity of white solid glycerides deposited. Their general characteristics are given in Table I.

Table I. *General characteristics of hen fats.*

	Yield of fat from adipose tissue %	Extracted fat			Mixed acids setting-point °C.
		Sap. equiv.	I.V.	Acid value	
From 24 hens (fish meal-free diet):					
Abdominal	97·5	285·5	80·1	0·7	34·5
Gizzard	96·9	286·0	78·8	1·7	35·1
Neck	96·0	285·9	79·8	1·4	34·6
From 12 hens (7 % fish meal in diet):					
Abdominal	96·4	286·3	78·5	0·7	36·2
Gizzard	95·8	284·5	79·7	5·6	36·5
Neck	88·7	284·8	77·4	1·2	36·5

The component acids present in each fat were separated by means of lead salts into "solid" and "liquid" acids, after which the methyl esters of each of these groups of acids were fractionally distilled in a vacuum. Analyses of the esters of the "solid" acids furnished quite normal results, but those of the esters of the "liquid" acids showed two unusual features. In the first place, the penultimate and the residual ester fractions in this group possessed analytical characteristics which indicated the presence of small amounts of unsaturated acids of the C_{20} and C_{22} series; the proportions present were approximately calculated as described by Banks and Hilditch [1932], who encountered a similar instance in the case of sow body fats. In the second place, the saponification equivalents of the most volatile fractions of the "liquid" esters (obtained by refractionation of the lowest-boiling primary fraction) were unusually low, and when the unsaturated material present therein was calculated on the assumption

[1] During the first 6 weeks the birds were in confinement and their ration included minute amounts of olive oil containing calciferol (0·001 ml. per bird daily); after 6 weeks the birds had access to free range.

Table II. *Complete analytical data for the abdominal and gizzard fats from the group of 24 hens.*

(i) Lead salt separation of mixed fatty acids.

	Abdominal layer fat		Gizzard fat	
	g.	%	g.	%
"Solid" acids S	91·1	31·8	89·6	31·1
"Liquid" acids L	195·0	68·2	198·6	68·9

(ii) Fractional distillation of the methyl esters of the "solid" acids (S).

No.	g.	Sap. equiv.	I.V.	g.	Sap. equiv.	I.V.
S 1	4·16	268·9	1·4	3·59	269·5	1·3
S 2	4·71	269·9	1·4	5·87	269·8	1·4
S 3	7·93	270·8	1·4	8·02	270·4	1·6
S 4	11·77	272·3	1·8	9·75	272·0	1·8
S 5	10·86	273·5	2·3	15·53	272·5	2·0
S 6	11·62	274·9	3·0	14·76	276·1	3·2
S 7	11·89	278·9	4·2	14·63	280·9	4·8
S 8	10·37	288·5	6·6	6·01	289·2	6·6
S 9	3·46	297·6	10·8	6·22	296·9	9·5
	76·77			84·38		

(iii) Fractional distillation of the methyl esters of the "liquid" acids (L).

No.	g.	Sap. equiv.	I.V.	g.	Sap. equiv.	I.V.
L 1	29·53	286·1	104·8	33·92	284·4	104·1
L 2	11·59	291·6	112·8	9·94	291·7	116·5
L 3	12·13	293·1	114·0	10·86	292·9	117·4
L 4	10·99	295·1	115·4	9·89	293·6	117·6
L 5	11·47	295·4	116·0	11·94	293·6	119·6
L 6	12·18	296·5	114·5	6·87	294·8	118·1
L 7	4·65	312·7	114·8	7·23	308·6	123·2
	92·54	(297·8)*	(116·0)*	90·65	(297·1)*	(119·9)*

(iv) Determination of saturated esters in fractions L 1.

Wt. oxidised (g.)	25·79	30·15
Wt. of saturated esters recovered (g.)	2·24	2·92
I.V. of saturated esters recovered	1·0	3·9
Sap. equiv. of saturated esters recovered	267·7	269·9

(v) Fractional distillation of the hydrogenated methyl esters of the "liquid" acids (HL).

No.	g.	Sap. equiv.	I.V.	g.	Sap. equiv.	I.V.
HL 1	2·83	279·0	—	4·86	280·2	1·2
HL 2	3·02	283·1	—	9·65	287·5	1·5
HL 3	6·10	287·2	—	12·00	290·6	1·9
HL 4	9·23	290·7	—	13·40	293·9	2·0
HL 5	11·77	293·3	—	14·33	295·8	2·1
HL 6	11·47	295·9	—	14·89	297·0	2·1
HL 7	13·88	296·3	—	9·18	298·8	1·9
HL 8	13·66	297·6	—	7·58	313·6	10·1
HL 9	7·02	299·0	0·3	—	—	—
HL 10	5·42	306·9	1·8	—	—	—
	84·40	(301·2)*	(1·7)*	85·89	(299·9)*	(6·9)*

* Saponification equivalents and iodine values of residual esters freed from unsaponifiable material.

that only unsaturated esters of C_{18} acids were present, the consequent equivalent of the saturated esters indicated the presence of methyl laurate or of esters of even lower molecular weight [*cf.* Grossfeld, 1930; 1931]. Careful qualitative examination of the acids present in these most volatile fractions showed on the other hand that the saturated acid actually present was mainly palmitic, probably accompanied by minor amounts of myristic acid; and no trace could be detected of acids of lower molecular weight than myristic acid.

The methyl esters of the "liquid" fatty acids were therefore divided into two halves, one of which was fractionally distilled without further treatment, while the other was similarly treated after converting it practically completely into saturated esters by hydrogenation in presence of a nickel catalyst at 170–180°. In the direct fractionation of the "liquid" esters, the first fraction collected (which was usually about one-third of the material distilled) was not submitted to further refractionation, but was oxidised in acetone solution with potassium permanganate in order, as far as possible, to remove all unsaturated esters. Consequently, as in the case of a rat body fat [Banks *et al.*, 1933], the mean equivalent and unsaturation of the unsaturated esters could then be calculated, after the proportion and mean equivalent of the saturated esters (which remain unaltered during the oxidation) had been determined.

When the hydrogenated portion of the "liquid" esters was fractionally distilled, it was found in all cases to contain comparatively large proportions of methyl palmitate and, indeed, much larger amounts of this ester than were isolated by the oxidation procedure to which reference has just been made. This fact, in addition to giving an alternative quantitative estimate for palmitoleic acid present (see below), affords definite proof of the existence in the original "liquid" esters of an unsaturated derivative of the normal C_{16} series of acids.

The experimental data from which the final compositions of the mixed fatty acids were calculated, by means of the two latter alternatives (oxidation of the primary "liquid" ester fraction or fractionation of the hydrogenated "liquid" esters), are illustrated in Table II. This gives the results obtained with the abdominal and gizzard fats from the group of 24 hens which had been reared on a fish meal-free diet.

From the proportions and equivalents of the saturated esters obtained by permanganate-acetone oxidation of the fractions L 1, the weights of the various esters present therein were estimated to be as follows:

Estimated composition of methyl ester fractions L 1.

	Abdominal layer fat (g.)	Gizzard fat (g.)
Myristate	0·19	Trace
Palmitate	2·37	3·14
Palmitoleate	7·18	10·02
Oleate	11·58	11·57
Linoleate	8·21	9·19

Apart from this, the calculation of the components of the various ester fractions followed the customary procedure, and it may suffice to record here the percentage compositions found for the respective "solid," "liquid" and "hydrogenated liquid" groups of fatty acids (Table III).

Table IV gives the corresponding data for the total component acids of the two fats, derived from a combination of the values found for the "solid" acids and for the "liquid" acids by direct ester fractionation (and determination of saturated esters in the lowest-boiling "liquid" ester fraction).

Table III. *Estimated percentage compositions of the "solid," "liquid" and "hydrogenated liquid" acids.*

	Abdominal layer fat % (wt.)	Gizzard fat % (wt.)
"Solid" acids S:		
Myristic	0·3	0·2
Palmitic	73·7	73·1
Stearic	22·0	22·7
Oleic	4·0	4·0
Unsaponifiable	Trace	Trace
"Liquid" acids L:		
Myristic	0·2	Trace
Palmitic	3·2	3·6
Palmitoleic	7·7	11·0
Oleic	55·9	51·6
Linoleic	31·9	33·1
C_{20-22} unsaturated	0·8	0·4
Unsaponifiable	0·3	0·3
"Hydrogenated liquid" acids HL:		
C_{16}	13·4	14·6
C_{18}	85·6	84·2
C_{20-22}	0·9	0·8
Unsaponifiable	0·1	0·4

Table IV. *Summarised data for component fatty acids of original abdominal and gizzard fats.*

Acid	Solid acids S	Liquid acids L	Total	Fatty acids (excluding unsaponifiable matter) % (wt.)	% (mol.)
		Abdominal layer fat.			
	(31·8 %)	(68·2 %)			
Myristic	0·11	0·14	0·25	0·3	0·3
Palmitic	23·42	2·15	25·57	25·6	27·3
Stearic	7·00	—	7·00	7·0	6·7
Palmitoleic	—	5·27	5·27	5·3	5·7
Oleic	1·27	38·13	39·40	39·4	38·2
Linoleic	—	21·77	21·77	21·8	21·3
C_{20-22} unsaturated	—	0·57	0·57	0·6	0·5
Unsaponifiable	—	0·17	0·17	—	—
		Gizzard fat.			
	(31·1 %)	(68·9 %)			
Myristic	0·05	Trace	0·05	0·1	0·1
Palmitic	22·73	2·46	25·19	25·2	26·9
Stearic	7·06	—	7·06	7·1	6·8
Palmitoleic	—	7·58	7·58	7·6	8·1
Oleic	1·25	35·55	36·80	36·9	35·6
Linoleic	—	22·81	22·81	22·8	22·3
C_{20-22} unsaturated	—	0·28	0·28	0·3	0·2
Unsaponifiable	0·01	0·22	0·23	—	—

Table V gives the corresponding figures for the mixed fatty acids, when the increments of acids in the "solid" group are combined with those for the "hydrogenated liquid" acids. In this case allowance has been made for the slight alteration in the proportions of the two groups, due to the proportionate increase in weight of the "liquid" acids as a result of hydrogenation.

Since hydrogenation of an unsaturated acid gives rise to the same number of molecules of the corresponding saturated acid, and since nearly all the

Table V. *Summarised data for combination of "solid" acids with "hydrogenated liquid" acids of abdominal and gizzard fats.*

Acid	Solid acids S	Liquid acids L	Total	Fatty acids (excluding unsaponifiable matter) % (wt.)	% (mol.)
	Abdominal layer fat.				
	(31·7 %)	(68·3 %)			
Myristic	0·11	—	0·11	0·1	0·1
Palmitic	23·35	9·18	32·53	32·5	34·9
Stearic	6·98	58·45	65·43	65·5	63·3
Oleic	1·26	—	1·26	1·3	1·2
C_{20-22}	—	0·59	0·59	0·6	0·5
Unsaponifiable	—	0·08	0·08	—	—
	Gizzard fat.				
	(30·9 %)	(69·1 %)			
Myristic	0·05	—	0·05	0·1	0·1
Palmitic	22·59	10·06	32·65	32·7	35·1
Stearic	7·01	56·36	63·37	63·6	61·4
Oleic	1·24	1·86	3·10	3·1	3·0
C_{20-22}	—	0·54	0·54	0·5	0·4
Unsaponifiable	0·01	0·28	0·29	—	—

palmitic acid in the original fats appears in the "solid" group which is common to both series of analyses, a practically independent value for the palmitoleic acid present can be derived by combining the molar percentages of palmitic acid in Table V with those of Table IV. The stearic acid present in the original fat is also entirely segregated in the "solid" group and (with the minor proportions of myristic and C_{20-22} acids observed in the results given in Table V) the acids remaining over must originally have been C_{18} unsaturated acids. The molar percentage of these is therefore now determined by difference (the relative proportions of oleic and linoleic acids being calculated on the basis of those observed in the C_{18} unsaturated esters actually obtained in the course of the analysis by the direct fractionation of the "liquid" esters). The corresponding deduced molar (and thence weight) percentages of the acids present in the original fats, thus derived from the results of the analysis of the "hydrogenated liquid" esters, are given in Table VI, and it will be seen that the palmitoleic acid figures in this table and those in Table IV are in good agreement.

Table VI. *Alternative calculation of component acids from the palmitic acid figures before (Table IV) and after (Table V) hydrogenation.*

Acid	Abdominal layer fat % (mol.)	whence % (wt.)	Gizzard fat % (mol.)	whence % (wt.)
Myristic	0·1	0·1	0·1	0·1
Palmitic	27·3	25·6	26·9	25·3
Stearic	6·7	7·0	6·8	7·1
Palmitoleic	7·6	7·0	8·2	7·6
Oleic	37·1	38·4	35·4	36·6
Linoleic	20·7	21·3	22·2	22·8
C_{20-22} unsaturated	0·5	0·6	0·4	0·5

We have examined several of the individual ester fractions obtained in order to satisfy ourselves as to the identity of individual acids present.

(*a*) The fatty acids from the residual ester fractions L 7, after removal of unsaponifiable matter, were brominated in dry ethereal solution, when a very small amount of an insoluble bromo-addition product was precipitated, which was insoluble in boiling benzene and which decomposed without melting at about 220–230°. These properties are characteristic of the polybromo-addition products of highly unsaturated acids of the C_{20} or C_{22} series.

(*b*) Two fractions (L 4 and L 5) of "liquid" esters which appeared to consist wholly of C_{18} unsaturated esters were examined, after hydrolysis, with reference to the unsaturated acids present:

The acids (2·14 g.) from L 4 yielded no bromo-addition products insoluble in ether, but gave 0·42 g. of a substance, sparingly soluble in light petroleum, which, after crystallisation from the latter solvent, melted at 114° and showed no depression in melting-point when mixed with the tetrabromostearic acid isolated in the same manner from the mixed acids of cottonseed oil.

The potassium salts of the acids from L 5 were oxidised with dilute ice-cold alkaline permanganate according to Lapworth and Mottram [1925, 1]. From the products of oxidation there were isolated: (i) a tetrahydroxystearic acid (Found: C, 62·0; H, 10·0 %. $C_{18}H_{36}O_6$ requires: C, 62·1; H, 10·3 %) sparingly soluble in hot, and insoluble in cold, water, M.P. 168–171° (unchanged on admixture with the tetrahydroxystearic acid, M.P. 173°, obtained from the linoleic acid of cottonseed oil); and (ii) the dihydroxystearic acid, M.P. 130–131° (unchanged when mixed with an authentic specimen from ordinary oleic acid).

(*c*) As already stated, the saturated acids present in the lowest-boiling fractions resulting from redistillation of the fractions L 1 were exhaustively examined by fractional crystallisation in several cases. Several crops of crystals were obtained, the least soluble of which melted at 60·5–62·0° (unchanged when mixed with palmitic acid), whilst the most soluble acids melted at 46–47°; since this melting-point was raised to 52·5–55·5° by admixture with palmitic acid and to 47·5–50·0° by admixture with myristic acid, whilst addition of lauric acid depressed it to below 35°, it is clear that the only saturated components present were the two former acids.

(*d*) Identification of the saturated C_{16} acid produced during hydrogenation of the "liquid" esters was also attempted, but the lowest-boiling ester fractions (HL 1, equivalent 279–280) already contained much stearate, with the result that complete isolation of palmitic acid could not be effected. However, crystallisation from alcohol of the mixed acids (2·77 g.) from an ester fraction HL 1 gave a least soluble crop of crystals (1·55 g., M.P. 61–63°) and a second crop (0·73 g.) which melted sharply at 55°. The latter crop, the melting-point of which was raised on admixture with an equal weight of either palmitic or stearic acid, was evidently the characteristic palmitic-stearic acid "eutectic" mixture, whilst the first crop was similarly shown by mixed melting-point tests to be a mixture of palmitic with an excess of stearic acid. No indication was obtained of the presence of any acids other than palmitic and stearic.

(*e*) Finally, after the quantitative analyses had been completed, a mixture of the abdominal (500 g.) and gizzard (100 g.) fats from the hens fed on a fish meal-free diet was hydrolysed and submitted to the lead salt separation. The "liquid" acids were converted into methyl esters, and the lowest-boiling fractions were refractionated several times in order to obtain a mixture of esters as free as possible from C_{18} unsaturated components.

The specimen finally obtained (14·8 g., sap. equiv. 270·5, I.V. 80·3) was submitted to oxidation (and re-oxidation) in acetone solution with potassium permanganate. Unchanged saturated esters (4·02 g., sap. equiv. 264·2, I.V. 2·8)

were recovered, which indicated that the ester fraction oxidised was composed of saturated (4·0 g.), palmitoleic (8·7 g.) and C_{18} unsaturated (2·1 g.) esters.

The acidic products from the two oxidations were united, completely hydrolysed, and the mixture of mono- and di-basic acids (9·1 g.) was separated by cooling their light petroleum solution at 0°. The insoluble crystalline dibasic acids (6·2 g.) were crystallised from water and finally from ethyl acetate, when the product (3·1 g.) melted at 95–98°[1] (unchanged on admixture with azelaic acid) and had an equivalent of 93·2 (azelaic acid requires 94·0). The maximum possible yield of azelaic acid from the 2·1 g. of C_{18} unsaturated esters present in the substance oxidised is about 1·4 g., so that azelaic acid must have been produced in quantity from the palmitoleic ester. Moreover, the material (2·9 g.) which remained in solution in light petroleum was recovered and distilled at atmospheric pressure, when a distillate (1·4 g.), which came over at 220–225°, and possessed an equivalent of 133·0, was obtained (*n*-heptanoic acid, mol. wt. 130, boils at 223°). The residue from this distillation was solid at the ordinary temperature and was probably mainly azelaic acid, which is not completely insoluble in ice-cold light petroleum.

The palmitoleic acid of the hen fats is therefore the same as that which has been identified in various marine animal oils, and possesses the constitution $CH_3.[CH_2]_5.CH:CH.[CH_2]_7.COOH$.

It has been necessary to go into the foregoing details in consequence of the somewhat unusual character of the mixed fatty acids of the bird fats; but we may now proceed to tabulate the final results obtained for the abdominal, gizzard and neck fats of the two groups of birds. Table VII shows the data for

Table VII. *Component fatty acids of depot fats from group of 24 Light Sussex hens (age 7 months).*

Acid	Weight percentages			Molar percentages		
	Abdominal	Gizzard	Neck	Abdominal	Gizzard	Neck
(i) Calculated from the direct analysis.						
Myristic	0·3	0·1	0·3	0·3	0·1	0·3
Palmitic	25·6	25·2	26·7	27·3	26·9	28·4
Stearic	7·0	7·1	5·9	6·7	6·8	5·7
Palmitoleic	5·3	7·6	6·6	5·7	8·1	7·1
Oleic	39·4	36·9	39·0	38·2	35·6	37·6
Linoleic	21·8	22·8	21·2	21·3	22·3	20·6
C_{20-22} unsaturated	0·6	0·3	0·3	0·5	0·2	0·3
(ii) Calculated from the results of analysis of the "hydrogenated liquid" esters.						
Myristic	0·1	0·1	—	0·1	0·1	—
Palmitic	25·6	25·3	—	27·3	26·9	—
Stearic	7·0	7·1	—	6·7	6·8	—
Palmitoleic	7·0	7·6	—	7·6	8·2	—
Oleic	38·4	36·6	—	37·1	35·4	—
Linoleic	21·3	22·8	—	20·7	22·2	—
C_{20-22} unsaturated	0·6	0·5	—	0·5	0·4	—

the fats from the group of hens whose diet included no fish meal, whilst Table VIII shows the corresponding figures for the fats from the 12 hens which received 7 % of fish meal in their diet.

[1] A small amount of suberic acid (M.P. 132–136°, mixed M.P. 134–136°) was present with the azelaic acid; this was probably formed by further oxidation of a little dihydroxypalmitic acid produced during the initial oxidation [*cf.* Lapworth and Mottram, 1925, 2].

Table VIII. *Component fatty acids of depot fats from group of 12 Light Sussex hens (age 2 years).*

Acid	Weight percentages			Molar percentages		
	Abdominal	Gizzard	Neck	Abdominal	Gizzard	Neck
(i) Calculated from the direct analysis.						
Myristic	1·2	0·6	1·2	1·5	0·8	1·4
Palmitic	24·0	25·4	24·5	25·5	27·0	26·1
Stearic	4·1	4·2	4·2	3·9	4·0	4·0
Palmitoleic	6·7	7·1	6·9	7·2	7·7	7·4
Oleic	42·5	43·0	42·8	41·0	41·5	41·2
Linoleic	20·8	18·4	20·4	20·3	17·9	19·9
C_{20-22} unsaturated	0·7	1·3	Trace	0·6	1·1	Trace
(ii) Calculated from the results of analysis of the "hydrogenated liquid" esters.						
Myristic	0·9	0·3	—	1·1	0·4	—
Palmitic	24·0	25·4	—	25·5	27·0	—
Stearic	4·1	4·2	—	3·9	4·0	—
Palmitoleic	8·5	8·0	—	9·1	8·6	—
Oleic	41·3	43·2	—	39·9	41·7	—
Linoleic	20·3	18·5	—	19·7	18·0	—
C_{20-22} unsaturated	0·9	0·4	—	0·8	0·3	—

Summary and discussion.

The present work has shown that hen body fats possess specific characteristics which distinguish them from those of quadrupeds and also from those of fishes or marine mammals. They fall into one of the two broad groups of depot fats which are found in non-aquatic animals [*cf.* Banks *et al.*, 1933], namely, the class in which the component fatty acids contain only about 30–35 % of saturated acids. They are definitely not of the more saturated type found in the pig, ox, sheep, horse, *etc.*, in which the proportion of stearic acid is variable but usually considerable. In the hen depot fats, about 65 % of the component acids belong to the C_{18} series, but these are mainly oleic (35–38 %) and linoleic (20–22 %) acids; no evidence of acids more unsaturated than linoleic has been observed in the C_{18} group.

The presence of unsaturated C_{20-22} acids (although in very small amounts), but still more the occurrence of notable proportions (7–8 %) of palmitoleic acid, causes these bird depot fats to possess features which are somewhat reminiscent of those of marine animal fats. Indeed, the component acids of the hen fats may be regarded as intermediate in character between those of the fats of land and marine animals, but they are much closer to such fats as those of the rodents than to those of aquatic fauna.

It may be pointed out that palmitoleic acid (apart from its presence in the fats of aquatic animals) has only been reported up to the present in hen and rat body fats and in the fats of diphtheria bacilli [Chargaff, 1933] and of lycopodium spores [Riebsomer and Johnson, 1933].

The presence of 65–68 % of unsaturated acids in the component acids of the depot fats of an animal with so high a body temperature as the hen affords another interesting example of the fact that body temperature is by no means the sole determining factor of the relative saturation of depot fats. At the same time, the almost identical compositions of the fats from three different parts of the body of the birds, one of these being situated much farther from the outer skin than the others, may be contrasted with the progressive change in relative saturation on passing, for example, from the perinephric fat of the pig to the fat

lying nearest to the skin; and here it may well be that the more uniform body temperature of the feathered animal leads to the absence of any regional difference in depot fat composition. The influence of the body temperature is thus to be regarded as a secondary factor which may modify the composition of depot fat; but the specific type of the latter seems to depend primarily on the biological species.

Amongst the saturated acids of the hen fats, myristic acid is present in little more than traces, and stearic acid forms only 5–7 % of the total component acids. It is very interesting to observe however that, despite the specific features which seem to distinguish hen depot fats, their palmitic acid content is exactly of the same order (25–30 mol. %) as that found in practically all land animal fats, whether relatively saturated or unsaturated, studied up to the present. It is perhaps well to emphasise once more the regularity with which palmitic acid falls within these limits in the depot fats of land animals of widely varying types, and also to point out that a palmitic acid content of 25–30 % is practically never met with in the fats of marine animals and is only approached in very few instances in vegetable seed fats (in fruit coat fats the presence of even higher proportions of palmitic acid is of course less uncommon [Hilditch, 1933]).

Finally, we have shown that the occurrence of 7–8 % of palmitoleic acid in the hen fats is not dependent on the diet received by the birds. Within the limits of the experimental analytical error, all the six depot fats are closely similar. The only general differences between the two groups are that the proportions of stearic and linoleic acids are possibly slightly lower, and those of myristic, oleic and the C_{20-22} acids perhaps somewhat higher, on the whole, in the fats from the older birds (whose diet included fish meal) than in those of the others. This observation is indeed probably of greater interest in relation to the marked difference in age of the two groups of birds (7 months and 2 years respectively) than to any slight difference in their diets. The characters of the depot fats are the same both in young and in mature birds, fed on similar rations.

All the foregoing observations of course refer to birds which have received a diet of which fat formed only a minor proportion (4 % or less).

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CXI. FERMENTATION OF SUGAR BY THE ROOT NODULE BACTERIA[1].

By ARTTURI ILMARI VIRTANEN, MANNE NORDLUND and ELIAS HOLLO.

From the Laboratory of the Foundation for Chemical Research, Helsinki, Finland.

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The fermentation of carbohydrates by the root nodule bacteria is a question of great interest, considering that the ability to fix atmospheric nitrogen must necessarily be dependent on the ability to ferment and utilise suitable carbon compounds. The solution of this question might possibly throw some light on the complicated problem of the mechanism of nitrogen fixation.

Although a vigorous fixation of nitrogen occurs when the nodule bacteria are grown in symbiosis with the appropriate host plant, recent investigations have lent additional support to the view that these bacteria are not capable of fixing nitrogen when grown apart from the host. Nor does any fixation occur when the nodules as such are separated from the host plant and cultivated on a synthetic medium, so that the power to fix atmospheric nitrogen cannot be attributable to any special difference in the structure of endonodular bacteria. In our opinion, all this leads to the assumption that the nodule bacteria receive from their host plant some particularly suitable form of carbon nutrition—probably labile carbohydrates, trioses or hexoses—which enables them to carry out the fixation process.

The utilisation of various carbon compounds by the nodule bacteria has been the subject of numerous and extensive investigations. In this respect, our knowledge has been particularly widened by the work of Baldwin and Fred [1927]. These investigations show that nodule bacteria actually utilise a great variety of carbohydrates and related compounds, although different strains differ considerably in this respect.

The decomposition of sugars by the nodule bacteria generally takes place very slowly. Aldohexoses seem to be more readily fermentable than fructose. The characteristics of the fermentation differ considerably for different strains, some strains causing the reaction of the medium to become acid, others alkaline. Very little is known, however, of the fermentation products formed, with the exception of the gum or slime which is obviously produced from carbohydrates.

As far as we know, the actual process of the fermentation of sugars by the nodule bacteria is totally unexplored. This is largely due to the fact that by the old technique hitherto employed—inoculation of the substrate solution with small amounts of the organism in question—the isolation and accurate determination of different fermentation products is scarcely possible owing to the very slow rate of the decomposition. We have therefore used in the present work the technique employed in this laboratory for several years, *i.e.* we have cultivated the organism separately on suitable media and used heavy suspensions

[1] A preliminary note on the subject appeared in the *Acta Chemica Fennica*, B 4, 62 (1932).

of the cell-mass thus obtained to act upon the substrate. The advantages of this method are obvious. The increased rate of sugar breakdown renders possible the identification and determination of the end-products, whilst the mechanism of the fermentation can be substantially elucidated by interrupting the process at different stages and determining the intermediate products present.

Experimental.

The bacterial species used was a laboratory strain of *Rhizobium trifolii* isolated from red clover and carried in stock for several years.

For the production of bacterial mass, 300–500 slant gelatin test-tubes were used at a time. The nutrient medium was prepared as follows:

Glucose	5·0 g.
Asparagine	2·5 g.
Gelatin	130 g.
Pea-extract	200 ml.
Tap-water	to 1000 ml.

The inoculated cultures were maintained at room temperature for 4 days, after which the bacterial mass was washed off with sterile water. This was done in an inoculation room where all necessary apparatus had been previously sterilised with a Hg-lamp. The suspension was centrifuged clear, and the bacterial mass was washed with warm sterile water. 100 test-tubes produced approximately 1·7 g. of bacterial mass which was practically free from gum.

A total of 20 fermentation experiments was carried out. Calcium lactate was used as substrate in two experiments and calcium butyrate in one, whilst glucose (1–6 g.) was used in all other experiments. In most cases, calcium carbonate was added to neutralise the acids formed during the fermentation. The fermentations were carried out under anaerobic conditions[1] attained by filling the fermentation flasks either with carbon dioxide or nitrogen. All fermentations were carried out at a temperature of 37°. The gases produced were collected and measured over mercury.

The evolution of gas set in, as a rule, about 30 hours after the addition of the bacterial suspension. A considerable portion of the gas was hydrogen.

The destruction of sugar was not complete. In several experiments only about one-half of the glucose originally present was fermented. In very few cases the destruction of sugar amounted to 75 % or more of the original. Sugar determinations were made by Bertrand's method, and the reducing sugar was identified as unfermented glucose by means of its phenylosazone.

After the fermentation had come to an end, the fermented liquid was tested for bacterial purity both microscopically and using gelatin plates. Analyses of the fermentation products were carried out in the manner generally employed in this laboratory.

In one experiment, sterile nodules were used instead of bacterial suspension as the fermenting agent. The nature of the fermentation thereby caused was similar to that caused by the bacterial suspensions.

Results.

It appeared that the relative proportions of fermentation products were closely dependent on the duration of the experiment. During the first stages of the process, considerable amounts of inactive lactic acid were formed from

[1] A preliminary experiment was also made aerobically. The presence of butyric acid in the fermented liquid was noted, but no accurate analysis of the end-products was made.

glucose. Lactic acid is, however, slowly fermented further with the production of butyric acid which is one of the end-products of the fermentation. In addition to lactic and butyric acids, small amounts of acetic acid, ethyl alcohol, carbon dioxide and hydrogen are also formed. The molecular ratio of butyric acid to carbon dioxide and hydrogen is: $C_4H_8O_2:CO_2:H_2=1:2:2$. The fermentation of glucose by the root nodule bacteria is thus a butyric acid fermentation.

Table I shows the results of a series of experiments. In each experiment equal amounts of glucose and $CaCO_3$ and equal amounts of the same bacterial mass were used, the duration of the experiments varying from 90 to 306 hours.

Table I.

Each flask contained 4 g. glucose, 5 g. $CaCO_3$, 2·2 g. moist bacterial mass, 250 ml. tap-water and was filled with nitrogen gas.

Exp. No.	10	11	12
Duration of experiment, hours	90	142	306
Glucose fermented, %	48·2	50·6	84·35
Alcohol, % of fermented glucose	5·25	2·4	6·55
Lactic acid, % of fermented glucose	22·6	18·2	3·0
Butyric acid, % of fermented glucose	24·2	27·3	35·45
Acetic acid, % of fermented glucose	2·9	4·05	Traces

For comparison, results from some other experiments are given in Table II.

Table II.

Exp. No.	7	9	14*
Duration of experiment, hours	96	90	90
Glucose fermented, %	72·2	49·2	29·0
Alcohol, % of fermented glucose	Traces	3·8	Not determined
Lactic acid, % of fermented glucose	40·2	21·4	26·8
Butyric acid, % of fermented glucose	13·0	31·8	30·5
Acetic acid, % of fermented glucose	4·2	Traces	5·1
CO_2, % of fermented glucose	16·0	Not determined	Not determined
H_2, % of fermented glucose	Not determined	Not determined	1·5

* The bacterial mass used in this experiment was somewhat too old and had lost a great part of its activity.

Identification and determination of different fermentation products.

Lactic acid. Quantitative determination of lactic acid was made using the Hirsch-Kauffman [1924] modification of the Fürth-Charnass method.

For identification, lactic acid was isolated from the fermentation liquid as zinc salt after the volatile acids had been distilled off with steam. The product was twice recrystallised from hot water and gave on analysis values consistent with the formula $(C_3H_5O_3)_2Zn, 3H_2O$ for the zinc salt of *dl*-lactic acid. (Found: H_2O, 18·19; ZnO, 26·87 %. $(C_3H_5O_3)_2Zn, 3H_2O$ requires H_2O, 18·18; ZnO, 27·37 %.)

Alcohol was determined according to Kuriloff [1897].

In order to identify the volatile neutral substance produced in the fermentation, the fermented liquid was subjected to steam-distillation at about p_H 8. The distillate was then acidified with sulphuric acid to p_H 2. The volatile substance was again distilled and oxidised with potassium dichromate and sulphuric acid in an autoclave at 110°. The acids thereby formed were determined according to Virtanen [1923] and Virtanen and Pulkki [1928]. It appeared that the oxidation yielded only acetic acid and that, consequently, the volatile fermentation product was ethyl alcohol.

Example. Determination of the original alcohol content according to Kuriloff gave 0·222 g. alcohol in the distillate. From this amount, 46·4 ml. of 0·1 *N* acetic acid were formed on oxidation, corresponding to 0·214 g. ethyl alcohol. The value for the semi-distillation was 36·3 %.

Carbon dioxide. In Exp. 7 the fermentation flask contained:

Glucose	3 g.
$CaCO_3$ (pure)	3·5 g.
Bacterial mass	3·0 g.
CO_2-free tap water	300 ml.

All air in the flask was replaced by CO_2-free nitrogen. The gases evolved were passed into KOH. After completed fermentation the flask was kept in a boiling NaCl solution for 1½ hours, a slow stream of nitrogen being passed through the apparatus. The weight of CO_2 obtained was 0·665 g.

On the basis of the figures presented in Table II the following calculation can be made:

0·091 g. acetic acid	liberates	0·0333 g. CO_2	from $CaCO_3$
0·871 g. lactic acid	,,	0·213	,,
0·281 g. butyric acid	,,	0·070	,,

Total amount of CO_2 liberated by acids = 0·3163 g.

Assuming that in the production of each molecule of acetic acid one molecule of CO_2 is split off—for 0·091 g. acetic acid 0·0667 g. CO_2—the amount of CO_2 formed in the butyric fermentation will be:

$$0{\cdot}665 - (0{\cdot}3163 + 0{\cdot}0667) = 0{\cdot}282 \text{ g.}$$

In the above experiment 0·281 g. butyric acid was formed, the ratio butyric acid : carbon dioxide being thus 1 : 2.

Attempts to measure the CO_2 production directly, using strong buffer solutions, failed.

Hydrogen. In Exp. 14, all air was carefully removed from the fermentation apparatus by means of carbon dioxide. The gases produced by fermentation were first led into a mercury burette and thereafter into a burette filled with 40 % KOH. The amount of hydrogen was 162 ml., *i.e.* 13·3 mg. of hydrogen at N.T.P., against 12·0 mg., calculated from the amount of butyric acid formed (0·265 g.).

Succinic and formic acids and glycerol. Succinic acid was determined only in Exp. 1, its amount being found to be 1·2 % of the glucose fermented. This small amount might possibly have been formed from the bacterial mass.

Glycerol is not produced by the nodule bacteria. The same is true of formic acid. Determinations according to Eds [1924–25] gave negative results.

Fermentation of lactic acid by the nodule bacteria.

This was studied in Exps. 6 and 15, using calcium lactate as substrate. It appeared that lactic acid itself is fermented very slowly. Thus, in Exp. 15 only 5 % of the lactic acid was decomposed in 4 days. In Exp. 6, 1·583 g. of pure lactic acid (neutralised with $CaCO_3$) were used together with 1·5 g. of moist bacterial mass. From this amount 1·077 g. were recovered after 11 days. The extent of destruction was thus only 28 %. Amongst the fermentation products were found 0·175 g. of acetic acid and 0·071 g. of butyric acid.

Recovery and identification of unfermented glucose in the fermentation liquid.

Exp. 18. The fermentation flask contained:

Glucose	3 g.
$CaCO_3$	3 g.
Bacterial mass	3·5 g.
Tap-water	250 ml.

The fermentation was carried out in an atmosphere of carbon dioxide. The evolution of gas ceased after 10 days. The volume of the gas was not measured.

The amount of glucose fermented was 2·765 g. or 92·2 %.

After completed fermentation the liquid was centrifuged, acidified with acetic acid and concentrated under reduced pressure to a volume of 95 ml. 30 ml. of the concentrate, containing 68 mg. sugar (as glucose), were heated on the water-bath with some $CaCO_3$ for half an hour. After cooling, 0·2 g. of fresh baker's yeast was added and the flask connected with a 10·0 ml. gas-burette. Temperature 37°. 17·5 ml. of gas were obtained in 23 hours and the fermented liquid did not reduce Fehling's solution. The 68 mg. glucose originally present in the concentrate would have given rise to about 33 mg. or 16·5 ml. of CO_2.

From the remaining 65 ml. of the concentrate the protein substances were removed by $HCl + HgCl_2$, the mercury was precipitated with H_2S and the hydrogen sulphide removed by a stream of air. The solution was then brought with NaOH to about p_H 5 and concentrated *in vacuo* to a volume of 30 ml. and treated with phenylhydrazine hydrochloride and sodium acetate on the water-bath. The phenylosazone formed was washed with hot water and further purified by recrystallisation from 50 % alcohol. It consisted of yellow needles which melted sharply at 205°.

The reducing substance in the fermentation liquid was thus unfermented glucose.

Experiment in which the bacterial mass was grown on liquid medium.

The growth of the nodule bacteria on liquid media is generally very slow, and it is therefore difficult to obtain considerable quantities of the bacteria from liquid cultures. An addition of mould-extract, however, promotes the growth of the nodule bacteria to a surprisingly great extent. The mould-extract was prepared by growing *Aspergillus niger* in suitable media and extracting the mycelium obtained with hot water at p_H 7.

The following experiment was, however, made with bacterial mass grown in an ordinary culture medium of the following composition:

KNO_3	0·5 g.	$FeCl_3$	Traces
K_2HPO_4	0·5 g.	$MnSO_4$	Traces
$MgSO_4, 7H_2O$ ...	0·2 g.	Yeast-extract ...	200·0 ml.
NaCl	0·2 g.	Tap-water ...	to 1000·0 ml.

After incubation for 7 days, the bacterial mass was centrifuged off, washed and suspended in a fermentation flask containing:

Glucose	3 g.
$CaCO_3$	3 g.
Tap-water	250 ml.

The weight of the bacterial mass obtained was 2 g.

The experiment was carried out in an atmosphere of CO_2 for 7 days. The amount of gas produced was 425 ml. The results were:

Glucose fermented ...	1·07 g.	= 35·7 %
Alcohol	0·039 g.	= 3·6 % of ferm. glucose
Butyric acid	0·443 g.	= 41·5 % ,,
Lactic acid	0·058 g.	= 5·5 % ,,

Other products were not determined.

Experiments with sterile nodules.

Twenty-four pea-plants, inoculated with *Rh. leguminosarum*, were grown in six Woulff-flasks in quartz sand under aseptic conditions[1]. After 2 months, the nodules were aseptically cut off from the roots, crushed in a sterile mortar, centrifuged off, washed with sterile water and suspended in a solution containing

Glucose	3 g.
$CaCO_3$	3 g.
Tap-water	250 ml.

The weight of the moist nodule-mass was 5 g. Development of gas ceased after 67 hours. The volume of gas was not measured. Plate culture on gelatin remained clear for over 3 days. Results:

Glucose fermented ...	0·945 g.	= 31·5 %
Alcohol	0·079 g.	= 8·4 % of ferm. glucose
Lactic acid	0·160 g.	= 17·0 % ,,
Acetic acid	0·115 g.	= 12·2 % ,,
Butyric acid	0·185 g.	= 19·6 % ,,

A comparison of the above results with those given in Tables I and II shows that the fermentation caused by separated nodules is, on the whole, very similar to that caused by bacterial suspensions, though in the former case slightly more acetic acid and alcohol were formed.

Catalase content of Rh. trifolii.

Determination of the catalase content of *Rh. trifolii* gave the following results:

$$k = 0{\cdot}00337, \text{ kat. v.} = \frac{0{\cdot}00337}{1350 \times 10^6} = 0{\cdot}0025 \times 10^{-9}.$$

The catalase content of the nodule bacteria is thus comparatively low. For comparison it may be mentioned that Virtanen and Karström [1925] and Virtanen and Winter [1928] found the following values for the catalase content of certain common micro-organisms:

B. fluorescens liquefaciens	$0{\cdot}08 \times 10^{-9}$
B. pyocyaneum	$0{\cdot}41 \times 10^{-9}$
Acetobacter suboxydans	$0{\cdot}01 \times 10^{-9}$
B. coli (10 strains)	$0{\cdot}004$–$0{\cdot}02 \times 10^{-9}$
B. acidi propionici	$2{\cdot}2 \times 10^{-9}$

[1] The method thereby employed has been described by Virtanen *et al.* [1933].

SUMMARY.

Fermentation of glucose by the root nodule bacteria was studied using heavy suspensions of *Rh. trifolii*, grown separately on a pea-extract gelatin medium.

The fermentation thereby caused is of the butyric type. During the early stages of the process considerable amounts of *dl*-lactic acid are formed from glucose. Lactic acid is, however, slowly fermented further with the production of butyric acid which is one of the end-products. In addition to lactic and butyric acids, carbon dioxide, hydrogen and small amounts of acetic acid and ethyl alcohol are also formed. The molecular ratio of butyric acid to carbon dioxide and hydrogen is $C_4H_8O_2 : CO_2 : H_2 = 1 : 2 : 2$.

Similar fermentation of glucose is also caused by crushed nodules from plants grown in sterile quartz sand cultures.

An aqueous extract of mould mycelium was found to stimulate markedly the growth of *Rh. trifolii*.

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CXII. THE METABOLISM OF NAPHTHALENE IN RABBITS.

By MARGHERITA COTONIO BOURNE and LESLIE YOUNG[1].

From the Department of Physiology and Biochemistry, University College, London.

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Interest in the metabolism of aromatic hydrocarbons has been stimulated by the recent work on the carcinogenic properties of certain members of this group of substances. But the problem of the metabolism of naphthalene is of especial interest because of the property which this substance possesses of being able to produce, when administered to rabbits in repeated doses, an opacity of the crystalline lens of the eye which, histologically, closely resembles human senile cataract [Bouchard and Charrin, 1886; Adams, 1930].

The work reported in the present paper forms part of a series of investigations directed towards the discovery of the mechanism by which naphthalene acts upon the organism so as to induce degenerative changes in the lens-proteins. At present nothing is known of this mechanism; it is still uncertain whether naphthalene or some derivative of it formed in the body acts directly on the lens, or whether the cataract is a secondary phenomenon resulting from some metabolic disturbance or toxic action upon a tissue remote from the eye. An essential step in the solution of this problem appeared to be to obtain some knowledge of the fate of ingested naphthalene. Therefore an attempt to identify the excretory products of naphthalene in the urine of rabbits has been made.

It is the purpose of the present paper to describe the isolation and identification of one metabolic product of naphthalene, *viz.* *l*-α-naphthylmercapturic acid; and to present evidence for the existence of a second metabolite, which has not been isolated, but which gives rise to naphthalene on acidification.

Experimental.

Buck rabbits weighing between 2 and 3 kg. and feeding on oats and cabbage were used exclusively. Naphthalene was dissolved in warm liquid paraffin and administered by means of a stomach tube. Doses of 1 or 2 g. were given to each rabbit and repeated at intervals of 2 or 3 days. The rabbits were kept in individual metabolism cages of suitable size, and the urine was collected separate from the faeces.

Colour tests for naphthols and glycuronic acid.

Certain authors [Igersheimer and Ruben, 1910; Edelfsen, 1905], on the basis of colour reactions applied to the urine excreted by dogs after naphthalene feeding, concluded that naphthalene was oxidised to naphthol in the body and was excreted in the urine as α- or β-naphthylglycuronate. Lesnick [1888] isolated a small amount of chemically pure α-naphthylglycuronate from dog's urine after the feeding of 12 g. of naphthalene. Penzoldt [1886] was unable to

[1] Bayliss-Starling Memorial Scholar.

obtain positive tests for naphthols, but presented evidence for the presence of naphthoquinone. Baumann and Herter [1877] obtained no naphthol on distillation of acidified naphthalene urine and reported that with steam-distillation only unchanged naphthalene came over.

We have not succeeded in isolating either naphthols or naphthylglycuronate from rabbit urine after dosing with naphthalene, but we have obtained positive diazo and ferric chloride reactions when these tests were applied to the residue from an ether extract of acidified naphthalene urine after hydrolysis. We have also obtained positive naphthoresorcinol tests for glycuronic acid. However, when these tests are applied to so complex a mixture as urine, the results cannot be interpreted with certainty, and we are led to conclude that, although naphthols may be present as ethereal sulphates or glycuronates, the amount is small and they do not appear to be the chief metabolic product of naphthalene in the case of the rabbit.

A compound giving rise to naphthalene on acidification.

The urine of rabbits after naphthalene feeding contains a soluble compound which yields naphthalene on acidification. This was demonstrated in the following manner. The alkaline urine excreted by a rabbit during the 24 hours following a 2 g. dose of naphthalene was collected. It was divided into two equal portions, each portion being placed in a conical flask. One portion was made acid with HCl, the other was kept as control and both were heated on a boiling water-bath. Within a short time colourless crystals began to collect on the cool parts of the flask containing the acidified urine; in the control flask no crystals appeared. The crystals were collected, washed with dilute acid, dilute NaOH and water; they were dried and resublimed. Their melting-point was found to be 79·5°; a mixture of these crystals and naphthalene also melted at 79·5°. A picrate melting at 149·5° was prepared from them. Their identity as naphthalene was thus established.

Naphthalene was not present in the alkaline urine, since none was sublimed out on heating the control flask, but was rapidly formed on acidification from some soluble precursor present in the urine. Efforts to isolate such a substance have so far been unsuccessful. The observation described might be accounted for by the presence in the urine of some compound such as dihydro-β-naphthol, a soluble compound which is rapidly converted to naphthalene by mineral acids [Bamberger and Lodter, 1895], and which might conceivably be formed in the body as a product of naphthalene metabolism.

l-α-*Naphthylmercapturic acid.*

This compound is not present in the urine of normal rabbits, nor does it occur after the feeding of liquid paraffin alone. It was found in the urine of rabbits after naphthalene feeding and was isolated in the following manner. The urine excreted by three rabbits during the 48 hours following the administration of 2 g. naphthalene to each rabbit was collected and mixed. It was made just neutral to litmus with HCl and then conc. HCl was added in the proportion of 10 ml. of acid for 100 ml. urine. The acidified urine was allowed to stand for about $\frac{1}{2}$ hour after which it was twice extracted with an equal volume of chloroform. The chloroform layer was collected and transferred to a distillation flask. Most of the chloroform was distilled off on a water-bath, and when the extract had been reduced to a small volume, it was transferred to an evaporating dish and the remainder of the chloroform carefully evaporated off with gentle heating on the water-bath.

The dark-brown, gummy residue so obtained was dissolved in a small volume of absolute ethyl alcohol. The alcohol solution was filtered if necessary and then diluted with 0·5 *N* HCl until opalescent. After standing in the ice-chest overnight a considerable bulk of precipitate consisting of colourless crystals mixed with amorphous brown material settled out. After this had been filtered off a second crop of crystals was sometimes obtained by further dilution of the filtrate.

The precipitate, after washing with water, was extracted with saturated barium hydroxide solution. The filtered baryta extract was acidified with HCl, whereupon an impure crystalline product separated out. This material was filtered off, washed with water and dried on a porous plate. The impurities present at this stage, which imparted a pinkish-brown colour to the material, possessed solubilities similar to those of the desired substance; further purification was therefore a matter of some difficulty. For this purpose the dried material was treated with a few ml. of cold 2 *N* NaOH; at first all the material went into solution, but upon brisk stirring the sodium salt of the compound separated out. This precipitate was filtered off immediately on a sintered glass filter and washed with a few ml. 2 *N* NaOH. The filtrate, which was coloured dark-brown, contained most of the contaminating substances. The sodium salt was dissolved in water; upon acidification of the solution the acid was precipitated as colourless crystals. Treatment with 2 *N* NaOH was repeated if the product obtained was more than faintly coloured.

The final stages of the purification consisted in repeated recrystallisations from aqueous alcohol until a colourless product of constant melting-point was obtained.

Amounts of l-α-*naphthylmercapturic acid present in urine.* From the 24 hours' urine of a single rabbit after 1 g. dose of naphthalene 52 mg. of the pure compound were obtained; 260 mg. of the pure compound were isolated from the mixed 24 hours' urine of three rabbits after the administration of 1 g. of naphthalene to each rabbit. Yields of between 300 and 400 mg. of the pure compound were obtained from the mixed 48 hours' urine of three rabbits after the administration of 2 g. of naphthalene to each rabbit.

Properties of l-α-*naphthylmercapturic acid.* It is an easily crystallisable, colourless, odourless solid; needles are obtained from aqueous alcohol; M.P. 170·5–171° (uncorr.). It is insoluble in water and in dilute mineral acids; soluble in alcohol, chloroform and pyridine; less soluble in ether, and only slightly soluble in hot benzene and toluene; soluble in dilute NaOH, barium hydroxide, sodium bicarbonate and dilute ammonia; completely soluble in conc. H_2SO_4 and when the solution is warmed a clear green colour develops. The compound is an acid and leaves no residue on ashing. When heated with soda-lime ammonia is easily split off. Carbon, nitrogen and sulphur were shown to be present; the test for ethereal sulphates was negative.

Analysis. After drying *in vacuo* at 80° over P_2O_5 the following analytical values were obtained (Schoeller). (Found: C, 62·14, 62·22; H, 5·23, 5·38; S, 11·24, 11·16; N, 4·75, 4·64 %. $C_{15}H_{15}O_3NS$ requires C, 62·28; H, 5·23; S, 11·09; N, 4·84 %.)

Mol. wt. By the camphor method, 311 (Schoeller); theory requires 289.

Neutralisation equivalent. The substance was dissolved in neutral alcohol and titrated with *N*/50 NaOH using phenolphthalein as an indicator. Found: 292; $C_{15}H_{15}O_3NS$ as a monobasic acid requires 289. The compound is therefore a monobasic acid.

Optical activity. The compound is laevorotatory; a 1 % solution in absolute ethyl alcohol has $[\alpha]_D^{25°} - 25°$.

Constitution of l-α-*naphthylmercapturic acid.*

Decomposition by acid. 0·669 g. of the substance was boiled for ¾ hour under reflux with 50 ml. dilute H_2SO_4 (12·5 % by volume). About half of the material remained undissolved and was removed by filtration of the hot solution. As the filtrate cooled slightly a small amount of crystalline material precipitated; this was also removed by filtration. The filtrate, while still warm, was made slightly alkaline to litmus by the cautious addition of concentrated ammonia. Upon cooling a precipitate appeared which separated out in crystalline form when the solution was allowed to stand in the ice-chest overnight. This precipitate was filtered off on a sintered glass filter; washed with water and dried at room temperature in the air; yield 0·183 g. This was recrystallised from hot water and a pure product in poor yield was obtained.

The recrystallised product was dried in air at 37° and had the following properties: insoluble in cold water, cold alcohol, ether, chloroform, and sodium bicarbonate solution; soluble in hot water, hot alcohol, dilute NaOH and dilute H_2SO_4; completely soluble in conc. H_2SO_4 and on warming the same green colour as is obtained with the parent substance developed. It had no definite melting-point but decomposed between 160 and 165°.

Analysis (Schoeller). (Found: C, 63·23, 63·08; H, 5·36, 5·36; S, 13·10; N, 5·44 %. $C_{13}H_{13}O_2NS$ requires C, 63·12; H, 5·30; S, 12·95; N, 5·66 %.)

A comparison of the empirical formula of this derivative with that of the parent substance showed that the two differed by C_2H_2O. It was therefore concluded that the effect of acid hydrolysis had been to split off one acetyl group from the molecule according to the equation:

$$C_{15}H_{15}O_3NS + H_2O \rightarrow C_{13}H_{13}O_2NS + CH_3COOH.$$

Acetyl estimation. The presence of one acetyl group in the molecule of the parent substance was proved by micro-acetyl estimation. (Found: 14·27, 14·21 % acetyl (Roth); theory requires 14·89 %.)

Decomposition by alkali. When boiled with dilute sodium hydroxide the compound was decomposed with the evolution of ammonia. Upon subsequent acidification of the solution a cloudiness appeared and after standing in the ice-chest, an oil separated out, which was soluble in alcohol and ether, gave a positive test for sulphur and had a characteristic unpleasant odour which suggested naphthylmercaptan. Since its low melting-point made it unsuitable for analysis, it was decided to oxidise it in the hope of obtaining a dinaphthyl-disulphide. Naphthylmercaptans are easily oxidised by the oxygen of the air in the presence of dilute sodium hydroxide.

0·498 g. of the parent substance was suspended in 50 ml. of 6 % NaOH and boiled for ¾ hour under reflux. The solution was then cooled, mixed with 50 ml. water and aerated for 48 hours. The solution soon became turbid, and a colourless solid separated out during the aeration period. After aeration was complete the mixture was allowed to stand in the ice-chest overnight to insure complete precipitation; the precipitate was filtered off, washed with water, and recrystallised from aqueous ethyl alcohol. In this way a product of definite melting-point, suitable for purification and analysis, was obtained.

It was a crystalline substance melting at 89°; soluble in ether and hot alcohol. Analysis (Schoeller): (Found: C, 75·60; H, 4·26; S, 20·17 %. $C_{10}H_7.S.S.C_{10}H_7$ requires C, 75·44; H, 4·43; S, 20·15 %). This substance is therefore identified as αα-dinaphthyldisulphide (M.P. 91°), the melting-point of the ββ-form being 139°.

The fact that αα-dinaphthyldisulphide could be isolated when alkaline decomposition of the compound was followed by aeration proves that α-naphthylmercaptan was one of the products of alkaline hydrolysis of the compound $C_{15}H_{15}O_3NS$.

Formula. On the basis of the information obtained by the acid and alkaline hydrolyses of the compound a probable constitutional formula can be deduced.

One acetyl group is present in the molecule, and the amphoteric character of the deacetylated compound suggests that the acetyl group is combined with nitrogen. Since one of the oxygen atoms is known to belong to an acetyl group, the possibility that the compound is a sulphonic acid is ruled out, and the acidic properties of the substance must be attributed to the presence of a carboxyl group.

That the compound is a naphthalene derivative is proved by the identification of α-naphthylmercaptan as one of the products of alkaline decomposition. The facts that the naphthalene nucleus is linked to the rest of the molecule through sulphur and that the linkage is in the α-position are also established by this means.

The following fragments have therefore been identified:

COOH, $NH.CO.CH_3$,

,

and of the whole molecule only C_2H_3 is left unaccounted for. On the basis of these facts the following constitutional formula is proposed:

This compound is an acetylated cysteine derivative, analogous to *p*-bromophenylmercapturic acid, which is excreted after bromobenzene feeding. So far as we are aware, it has not been previously described.

Discussion.

It has been shown that naphthalene is detoxicated by the rabbit partly by conjugation with cysteine and excretion as the mercapturic acid and partly by conversion into a soluble compound, not yet identified, which yields naphthalene on acidification. It is possible that other naphthalene derivatives, such as ethereal sulphates or phenolic substances are also excreted.

The evidence presented for the suggested constitutional formula of *l*-α-naphthylmercapturic acid has been confirmed by the synthesis of this compound, which is described in the following paper [Ing *et al.*, 1934].

Two interesting suggestions arise out of the observations here reported. In the first place conversion into the mercapturic acid may be a general method of detoxication of aromatic hydrocarbons in the animal body. Callow and Hele [1926] presented evidence that benzene as well as bromobenzene is excreted as the mercapturic acid, although the phenylmercapturic acid was not isolated. Secondly the fact that naphthalene requires cysteine for its detoxication suggests the possibility that the cataract which naphthalene produces may result from depriving the lens of cysteine. The β-krystallin of the lens is a cysteine-rich protein; glutathione and possibly free cysteine are present in the lens in relatively large amounts; furthermore the lens, which is an avascular tissue and

dependent on the aqueous humour for its supply of oxygen, is thought to have a specialised oxidation mechanism in which the cysteine-containing substances play an important rôle. It therefore seems likely that repeated doses of naphthalene, constituting a drain on the cysteine supplies in the body, might interfere with the metabolism of the lens by depriving it of cysteine; and thus the degenerative changes resulting in cataract might be induced.

Summary.

An organic acid, $C_{15}H_{15}O_3NS$, identified as *l*-α-naphthylmercapturic acid, has been isolated from the urine of rabbits after naphthalene dosing; the isolation and identification are described.

A second compound, a soluble substance yielding naphthalene on acidification, was also found to be present; this has not been isolated.

We wish to thank Prof. J. C. Drummond and Dr H. Raymond Ing for their helpful criticism and valuable advice. One of the authors (M. C. B.) is indebted to the Committee on the Physiology of Vision for a personal grant and for a grant which has defrayed the expense of this research.

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CXIII. SYNTHESIS OF *l*-α-NAPHTHYL-MERCAPTURIC ACID.

By HARRY RAYMOND ING, MARGHERITA COTONIO BOURNE and LESLIE YOUNG[1].

From the Departments of Pharmacology and of Physiology and Biochemistry, University College, London.

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In the preceding paper the isolation of an acid $C_{15}H_{15}O_3NS$ from the urine of rabbits fed with naphthalene was described and evidence presented which identified it with reasonable certainty as *l*-α-naphthylmercapturic acid, $C_{10}H_7S.CH_2.CH(NHAc).CO_2H$. In order to put this identification beyond doubt, the synthesis of this acid was attempted and has now been achieved.

The best method of synthesising mercapturic acids appeared to be the condensation of the appropriate aryldiazonium salt with acetylcysteine. Diazonium salts, ArN_2X, condense with thiol compounds to form $ArN_2S.R$, which decompose when heated in a suitable solvent, evolving nitrogen and forming thio-ethers, Ar.S.R [Kalle and Co., 1907]. Friedmann [1904] prepared *p*-bromophenylmercapturic acid in this way by condensing *p*-bromophenyldiazonium chloride with cysteine and acetylating the *p*-bromophenylcysteine so obtained. In our experiments α-naphthyldiazonium acetate was condensed with *d*-acetylcysteine and the condensation product decomposed in hot water. *l*-α-Naphthylmercapturic acid was obtained in poor yield and found to be identical with the mercapturic acid isolated from rabbits' urine.

Experimental.

d-Acetylcysteine was prepared by the method of Pirie and Hele [1933]. It melted at 108° (uncorr.) and a solution in water had $[\alpha]_D^{21°}$ 5·5° (c=2·7 %).

α-Naphthylamine (3·2 g.), recrystallised from light petroleum, was dissolved by warming in water (60 ml.) containing concentrated hydrochloric acid (2·5 ml.). The solution was cooled in ice with stirring and a further 2·5 ml. of concentrated hydrochloric acid and 30 g. of ice added. This suspension of α-naphthylamine hydrochloride was diazotised at 0° with a solution of sodium nitrite (1·6 g.) in water (15 ml.), which had been rendered acid to Congo paper by the addition of very dilute ice-cold hydrochloric acid [Bulow and Sprosser, 1908]. Sodium acetate (4 g.) in the least amount of water was then added to neutralise the hydrochloric acid and the solution filtered to remove a small amount of diazoaminonaphthalene. The filtrate was added slowly to a 10 % solution of *d*-acetylcysteine (3·2 g.) in water. An orange-coloured amorphous precipitate was formed, which was collected, suspended in water containing a few per cent. of alcohol and heated with the addition of copper-bronze powder on the water-bath until the evolution of nitrogen ceased. The hot solution was filtered, the

[1] Bayliss-Starling Memorial Scholar.

copper powder and insoluble material washed with boiling water and the united filtrates evaporated to small bulk. The semi-crystalline solid material which separated was collected and dissolved in potassium carbonate solution, and the solution was filtered from resinous material and acidified. The crude mercapturic acid, which was precipitated, was extracted with chloroform, and the solid residue from the evaporation of the chloroform was crystallised by dissolution in a little alcohol and, after treatment with charcoal, addition of water. Yellowish crystals were obtained melting at 169–170°.

The crude acid (0·25 g. from 5 g. of acetylcysteine) was purified by conversion into its sodium salt which, like the sodium salt of the natural mercapturic acid, crystallises from excess of 2 *N* sodium hydroxide. The free acid was recovered by acidifying an aqueous solution of the sodium salt and was crystallised from aqueous alcohol. This process was repeated once more and the acid finally crystallised from ethyl malonate, from which it separated in well-formed but still faintly coloured needles; yield 0·13 g. (Found: C, 62·30; H, 5·24; N, 4·60; S, 10·95 %. $C_{15}H_{15}O_3NS$ requires C, 62·28; H, 5·23; N, 4·84; S, 11·09 %.)

The synthetic α-naphthylmercapturic acid melted at 170–171°, the natural *l*-α-naphthylmercapturic acid at 170·5–171° and a mixture of the two at 170–171°.

The natural mercapturic acid in 1 % solution in alcohol had $[\alpha]_D^{22°} -25°$ and the synthetic acid in 1·005 % solution in alcohol had $[\alpha]_D^{22°} -23·9°$.

The synthetic *l*-α-naphthylmercapturic acid, like the natural product, was soluble in conc. H_2SO_4 and gave a green colour on warming.

Summary.

l-α-Naphthylmercapturic acid has been synthesised and proved to be identical with the mercapturic acid isolated from the urine of rabbits fed with naphthalene.

One of us (M. C. B.) is indebted to the Committee on the Physiology of Vision of the Medical Research Council for grants.

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CXIV. A NOTE ON THE REDUCING ACTIVITY OF THE TISSUES OF NORMAL AND TUMOUR-BEARING RATS AND MICE.

By ARTHUR FREDERICK WATSON and MICHELE MITOLO.

The Imperial Cancer Research Fund, London, and The Institute of Human Physiology, the Royal University, Rome.

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Harris and his colleagues have recently studied the reducing activity of acid extracts of a number of animal and vegetable tissues, and have found a correlation in most cases between this activity and the antiscorbutic potency of the tissues. Whether their technique [Birch, Harris and Ray, 1933] affords a specific test for such potency is still a matter of controversy but the authors themselves recognise the limitations of the method unless confirmed by biological assay. During the examination of the tissues Harris [1933, 1, 2] found that an extract of the Jensen rat sarcoma had a high reducing activity although he found by biological tests on guinea-pigs that this activity could not be attributed entirely to its ascorbic acid content. Similarly Borghi and Deotto [1934] were unable to demonstrate any antiscorbutic activity in extracts of the Ehrlich mouse carcinoma. Boyland [1933] confirmed the high content of reducing substances in the Jensen rat sarcoma but found a lower content for a second rat sarcoma, two mouse sarcomas, a mouse carcinoma (C 63) and a fowl sarcoma. In the case of the Jensen sarcoma but not in the other five tumours, the activity of the malignant tissues was higher than that of other tissues of the same animal. In commenting on Harris' results, Quastel [1933] cited in confirmation the greater reduction of ammonium molybdate by acid extracts of human tumours than by those of non-malignant tissues.

These observations have been extended in the present study to a number of different strains of transplantable rat and mouse tumours which have been propagated over a long period of years in the laboratories of the Imperial Cancer Research Fund. The reductions of both ammonium molybdate and 2:6-dichlorophenolindophenol by the tumours and other organs have been examined. The rats were maintained on a mixed diet of bread, meat biscuits, boiled beans, maize and kitchen scraps, and the mice on bread and oats, with cod-liver oil and yeast three times a week.

The reduction of ammonium molybdate by tissues of tumour-bearing rats and mice.

The following method was adopted for estimating the relative reducing activities of the tissues.

The tumour-bearing animal was killed and equal quantities of the different tissues ground up with dilute acetic acid and purified silver sand in hard glass centrifuge pots. 20 % ammonium molybdate was then added in equal amounts to each of the pots, which were incubated at 37° or 50° for 60 or 90 minutes.

The best results were usually obtained when 2 ml. of 3*N* acetic acid and 8 ml. of 20 % ammonium molybdate per 0·2 g. of tissue were used.

The intensity of the blue colour of the supernatant fluid after centrifuging was employed as a criterion of the degree of reduction of the molybdate.

Two rat tumours and eight mouse tumours were examined by this method and the results are shown in Table I.

Table I. *The reduction of ammonium molybdate by the tissues of rats and mice with transplantable tumours.*

		Tumour			Relative reducing values							
No.	Animal	Strain	Type	Age in days	Liver	Spleen	Brain	Muscle	Kidney	Lung	Necrotic tumour tissue	Non-necrotic tumour tissue
1	Rat	Jensen	Sarcoma	22	3	2	—	1	—	—	—	5
	,,	,,	,,	19	2	—	2	—	—	—	—	4
2	,,	Walker	Carcinoma	38	2	2	4	—	2	½	—	5
1	Mouse	Crocker	Sarcoma	15	2	½	—	0	—	—	—	4
2	,,	37	,,	14	2	3	—	1	—	—	—	4
3	,,	Bonne	Tar sarcoma	21	3	4	—	1	—	—	—	2
4	,,	173	,,	28	4	3	5	—	1	—	—	4
5	,,	2146	Tar carcinoma	20	2	4	—	1	—	—	—	4
	,,	2146	,,	15	1	2	—	½	—	—	—	3
6	,,	91	Carcinoma	66	5	4	—	1	1	—	—	4
7	,,	113	,,	28	2	3	—	½	1	—	½	4
8	,,	206	,,	18	2	3	4	—	—	—	1	—

The intensity of the blue colour was determined by comparison with an arbitrarily chosen standard, and the data in the right-hand columns afford an approximate comparison of the reducing activities of the tissues. The Jensen rat sarcoma and the Walker rat carcinoma showed activities of a higher order than the other tissues of the same animals. In the majority of the mouse tumours a similar increased activity of the malignant tissues was found. Exceptions were the Bonne and the 173 sarcomas and the 91 carcinoma, where the spleen, brain and liver respectively showed greater reducing activities. A slight reduction of the ammonium molybdate was effected even by the highly necrotic portions of a tumour as was shown in the case of tumours 113 and 206. Both the spleen and brain showed high reducing activities and in most cases these organs were superior to the liver in this respect.

The reduction of 2:6-dichlorophenolindophenol by acid extracts of the tissues of normal and tumour-bearing rats and mice.

A slight modification of the technique of Birch *et al.* [1933] was employed for testing the reducing activity of the extracts.

The animals were killed and the organs removed. 2 ml. of 10 % ice-cold trichloroacetic acid were added to small weighed quantities of each of the different organs which were then ground with 0·5 g. of purified silver sand in small hard glass centrifuge-pots. 2 ml. of cold distilled water were added in each case, and the material was centrifuged for 5 minutes. After centrifuging, the supernatant acid extracts were poured off into labelled beakers. 4 ml. of distilled water were added to the mixture of sand and tissue in each case and the centrifuging was continued for a further 5 minutes. This second supernatant was then added to the contents of the corresponding beaker. A third washing was found to be unnecessary. The 8 ml. of extract and washings contained therefore the acid extractives of the original weighed tissue.

These extracts were then titrated against a measured volume of the 2:6-dichlorophenolindophenol indicator. The indicator solution was made by dissolving 0·05 g. of the dried substance in a small quantity of boiling distilled water and filtering. The undissolved residue was extracted with a further quantity of boiling water and the total volume of the filtrate made up to 50 ml. The tissue extracts under examination were run from a micro-burette into 1·0 or 0·5 ml. of a 1 in 10 dilution of the indicator as prepared above, in a small beaker. Table II shows the reducing activities of the various tissues. The figures represent the ml. of the indicator solution (1 ml. of which was equivalent to 0·04 mg. of a commercial sample of ascorbic acid) reduced by the extract from 1 g. of tissue.

Table II. *The reducing activity of the acid extracts of the tissues of normal and tumour-bearing rats and mice.*

		Tumour			Relative reducing activities of tissues ml. 2:6-dichlorophenolindophenol solution (1/10,000) reduced by extract from 1 g. of tissue								
No.	Animal	Strain	Type	Age in days	Liver	Spleen	Brain	Kidney	Lung	Testes	Embryo	Necrotic tumour tissue	Non-necrotic tumour tissue
1	Rat	Jensen	Sarcoma	31	5·2	8·7	8·7	3·5	—	—	—	—	10·7
	,,	,,	,,	8	8·2	8·8	7·8	5·7	—	—	—	—	11·2
	,,	,,	,,	26	—	—	8·3	—	—	—	—	—	10·8
2	,,	Walker	Carc.	39	7·6	9·4	9·7	—	8·4	—	—	6·0	14·5
	,,	,,	,,	80	4·9	7·5	6·6	3·0	—	—	—	—	13·3
	,,	,,	,,	21	3·8	6·9	4·9	3·1	5·2	—	—	—	10·7
	,,	Normal	—	—	10·0	8·7	8·0	5·3	5·7	8·0	—	—	—
	,,	,,	—	—	10·0	9·7	—	5·8	8·2	7·4	—	—	—
	,,	,,	—	—	9·1	9·1	8·6	5·7	3·7	8·4	—	—	—
1	Mouse	Crocker	Sarcoma	21	10·5	14·5	14·3	9·0	13·3	—	—	—	14·1
2	,,	37	,,	13	9·5	8·3	16·3	5·0	8·0	—	—	—	7·2
	,,	37	,,	25	8·4	8·2	12·3	7·5	—	—	—	—	9·2
3	,,	Bonne	Tar sarc.	7	7·3	—	11·6	—	—	—	—	—	10·0
4	,,	173	,,	15	8·5	7·6	13·1	—	8·5	—	8·0	—	6·7
	,,	173	,,	22	5·7	10·0	12·5	—	6·9	—	—	—	6·7
5	,,	2146	Tar carc.	9	10·5	11·2	11·1	—	—	—	—	—	9·5
6	,,	113	Carc.	80	8·3	13·0	14·0	—	12·0	—	—	—	14·3
7	,,	4108	Sporadic	—	9·5	16·3	13·5	8·0	—	—	—	11·9	16·0
	,,	Normal	—	—	12·3	16·0	15·7	9·6	15·8	—	—	—	—
	,,	,,	—	—	8·9	10·4	14·8	7·0	14·0	—	—	—	—
	,,	,,	—	—	9·0	12·7	17·0	7·6	9·3	—	—	—	—

Both the Jensen and Walker tumours showed greater activity than the other tissues of the same animals. The variation in the reducing values of the rat tumours was small (minimum 10·7; maximum 14·5). There was a greater variation in the reducing values of the different strains of mouse tumours (minimum 6·7; maximum 16·0) and in most cases the values were of a lower order than those for the spleen or brain. The high reducing activity of the brain tissue is of interest in connection with some experiments which one of us (M. M.) has carried out in collaboration with Dr F. G. Young in the Biochemistry Department at University College, London [Young and Mitolo, 1934]. The general properties of the reducing substance prepared from ox brain clearly differentiate it from ascorbic acid and in this respect it shows a similarity to the reducing substance in tumour tissue [Harris, 1933, 2; Borghi and Deotto, 1934].

Tumour 4108 was the 6-weeks old autoplast of a spontaneously occurring mammary carcinoma. Both the non-necrotic and the necrotic portions had high reducing values. The values for the tissue extracts of normal tumour-free rats and mice were of a similar order to those of the tumour-bearing animals.

Although there is a considerable variation in the reducing values of the transplantable tumours of different strains, the values of tumours of the same strain show less variation even under different conditions. This is shown by the similarity of the values for (*a*) tumours of the same or different ages, (*b*) multiple transplantable tumours of the same animal, and (*c*) progressing and spontaneously regressing transplantable rat tumours.

Tumours of the same and different ages. In a series of nine 2146 mouse carcinomas the reducing values were 10·0, 9·0, 8·7, 8·5, 9·5, 11·0, 10·2, 9·4 and 9·5 after the same period of growth and before the tumours showed any necrosis. The values for the Jensen and Walker rat tumours and the mouse tumours 37 and 173 of different ages were very similar (Table II).

Multiple transplantable tumours. In cases where two transplants were made in the same animal the reducing values of the subsequent tumours were approximately the same, *e.g.*:

		Relative reducing activities	
		Left tumour	Right tumour
2146 (mouse tar carcinoma)	1.	9·5	9·2
	2.	10·2	10·0

Progressing and spontaneously regressing tumours. In two cases Jensen rat tumours which were showing rapid spontaneous regression were found to have reducing values of 10·9 and 12·5. These values were very similar to those found for the normal progressing tumours (10·7, 11·2, 10·8, Table II).

Summary.

1. Transplantable rat and mouse tumours of different strains readily reduce ammonium molybdate in an acid medium. In most cases the tumours show a higher content of reducing substances than the liver, spleen or brain.

2. The reducing substances in these tumours are soluble in dilute trichloroacetic acid, and extracts obtained in this way reduce 2:6-dichlorophenolindophenol. In the case of the rat strains the tumour extracts were more active than those of the liver, spleen or brain. They were somewhat less active than these extracts however in the case of the mouse strains.

3. Although there is a considerable variation in the reducing activities of the tumours of different strains, the values of the tumours of the same strain are comparatively constant. This is shown by the similarity of the reducing values for (*a*) tumours of the same and different ages, (*b*) multiple transplantable tumours in the same animal, and (*c*) progressing and spontaneously regressing tumours.

We are indebted to our colleague Dr Cramer for a number of the tumours used in these studies and to Mr H. E. Adams for help with the analyses.

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CXV. THE FORMATION OF COPROSTEROL IN THE INTESTINE[1].

I. POSSIBLE RÔLE OF DIHYDROCHOLESTEROL, AND A METHOD OF DETERMINING DIHYDROCHOLESTEROL IN PRESENCE OF COPROSTEROL.

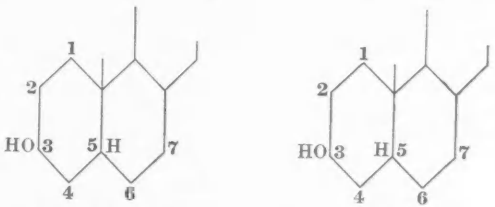
By HENRIK DAM.

From the Department of Chemistry, Institute of Pathology, Freiburg, Br., and the Biochemical Institute, University of Copenhagen.

(*Received April 28th, 1934.*)

Cholesterol of exogenous or endogenous origin is eliminated with the faeces mainly in the form of coprosterol. Soon after the discovery of this compound by Bondzynski and Humnicki [1896–97] it was suggested that coprosterol is formed by bacterial hydrogenation of cholesterol in the intestine, but no definite proof of this theory has as yet been forthcoming. All attempts to convert cholesterol into coprosterol by bacteria *in vitro* have been unsuccessful.

Human faeces contain, besides coprosterol, small quantities of unchanged cholesterol and dihydrocholesterol [Windaus and Uibrig, 1915]. Schönheimer *et al.* [1930] have shown that dihydrocholesterol is formed from cholesterol in the body tissues (or synthesised there) and is continuously secreted into the intestine, but intensive search for coprosterol in the organs has failed to reveal it even in traces. It is possible that the intestinal bacteria effect the stereochemical inversion of the secreted dihydrocholesterol at carbon atom 5 rather than the hydrogenation of the double bond in cholesterol. Were this the case, ingested dihydrocholesterol should, at least in part, reappear as coprosterol



Dihydrocholesterol (abbreviated) Coprosterol

in the faeces. Feeding experiments designed to test this view can be carried out approximately quantitatively because neither coprosterol nor dihydrocholesterol can be absorbed from the intestine [von Behring and Schönheimer, 1930].

To this end a method for the quantitative determination of dihydrocholesterol in presence of coprosterol is necessary. As these two isomerides closely

[1] This work was aided by a grant from the Josiah P. Macy, Jr., Foundation.

resemble each other in physical and chemical properties, few ways of doing this offer themselves. At first an attempt was made to estimate the amount of both sterols in a mixture with the aid of the fact that they are converted by sodium ethylate (or sodium in xylene) into the non-precipitable isomerides (epicholestanol and epicoprosterol) to a different extent. This method proved to be impracticable for analytical purposes as the values were not well reproducible. The method finally adopted involves the differential solubility of the digitonides of the two substances [Schönheimer and Dam, 1933]; coprosterol digitonide dissolves to the extent of 2 g. in 100 ml. of methyl alcohol, while the corresponding solubility of dihydrocholesterol digitonide is only 0·35 g. A curve can, therefore, be constructed whereby the composition of a mixture of the two can be determined directly from its solubility in methyl alcohol.

With the aid of this method it has been possible to show that dihydrocholesterol given by mouth is excreted unchanged and is not converted into coprosterol.

Experimental.

1. *Curves for the solubility of mixtures of the two sterol digitonides in methyl alcohol.*

The digitonides were prepared by precipitation of 0·5 % alcoholic solutions of the sterol mixtures with digitonin (50 % excess, time of precipitation 24 hours). On account of the solubility of coprosterol digitonide in ethyl alcohol, the precipitates were washed with 20 ml. of a saturated solution of coprosterol digitonide in 96 % alcohol and finally with 1 ml. of ether. For the solubility determination, quantities of about 135 mg. of the digitonide (dried at 110°) were weighed out in small flat-bottomed glass vessels and again dried; a loss of a few mg. was observed owing to the hygroscopic character of the substance. Then enough of the product was taken out to leave $127 \pm 0{\cdot}5$ mg. in the vessel, which was placed in a narrow, stoppered glass cylinder and covered with a rubber cap, and the cylinder shaken overnight in a thermostat at $25 \pm 0{\cdot}1°$. The solution was then filtered and the dry residue determined in 2 ml. of the filtrate. Great care must be taken that the methyl alcohol is dry, for the least trace of moisture depresses the solubility of the digitonide. The methyl alcohol for these experiments was dried by boiling for 20 minutes with calcium turnings and distilled with the exclusion of moisture by means of a calcium chloride tube. Extreme precautions were also taken to avoid contamination by moisture during storage.

Since the separation of the saturated sterols from cholesterol by the method of Schönheimer involves preliminary treatment with bromine, a solubility curve was also constructed of the digitonides which had been precipitated in alcohol containing sufficient bromine to give it a yellow colour. The presence of bromine slightly raised the solubility of the digitonides (curve 2, Fig. 1). All the values were obtained with the same digitonin preparation, for digitonins of different sources are known to possess slightly different properties.

An attempt was made by this method to estimate dihydrocholesterol added to the mixture of saturated sterols isolated from faeces. The saturated sterols from the faeces of a human subject whose diet contained very little plant sterol were isolated after bromination by the method of Schönheimer [1930]. The resulting digitonide had a solubility of 43·8 mg. in 2 ml. Since pure coprosterol digitonide has a solubility of 41·7 mg. (when precipitated in presence of bromine) this finding indicates that traces of an unknown more soluble substance were present in the mixture. Attempts to isolate a more soluble saturated fraction

by fractional precipitation with digitonin of the mother-liquor from the preparation of coprosterol led only to a digitonide with a maximum solubility of 46·7 mg. in 2 ml. of methyl alcohol. In the feeding experiments a special curve (No. 3 in Fig. 1) was therefore employed which related not to the content of dihydrocholesterol in a mixture with pure coprosterol but to the composition of mixtures of dihydrocholesterol and the saturated sterol of faecal origin.

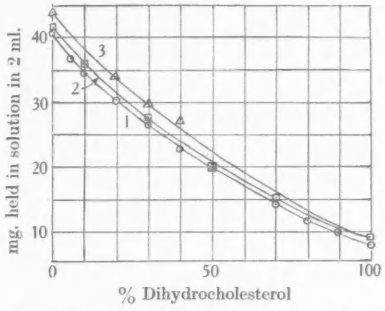


Fig. 1. Solubility of digitonides from coprosterol and dihydrocholesterol in absolute methyl alcohol at 25°, 127 mg. digitonide + 5 ml. methyl alcohol. 1. Digitonides precipitated without bromine. 2. Digitonides precipitated in presence of bromine. 3. Digitonide of sterol from human faeces + dihydrocholesterol, precipitated in presence of bromine.

The experimental findings are reproduced in Fig. 1. With the aid of these curves good recovery of added dihydrocholesterol was made from both coprosterol and the sterols of faeces. By means of curve 1 it was found that on hydrogenation of 0·2 g. allocholesterol with 0·2 g. of platinum oxide with hydrogen in cold ether, 55 % of coprosterol and 45 % of dihydrocholesterol were produced. The solubility of the digitonides of the reaction product was 23·8 mg. in 2 ml. of methyl alcohol.

2. *Feeding experiments with dihydrocholesterol.*

A healthy individual was kept on a constant diet of which the sterols were almost entirely of animal origin. The diet for each period consisted of 930 g. butter, 400 g. 30 % cream, 280 g. lean pork, 500 g. potatoes, 100 g. barley porridge, 732 g. zwieback and 40 g. Graham bread. During the experimental period he received a supplement of dihydrocholesterol approximately corresponding to the amount of total sterols excreted in the faeces. The total and saturated sterols were determined in the stools during a pre-experimental, an experimental and two post-experimental periods, and the solubility of the saturated sterol digitonides was measured. Each period occupied 5 days, of which the last was made a fast day in order to obtain a better differentiation of the periods (the day before the beginning of the experiment was also a fast day). The beginning of each period in the faeces was marked by a carmine tablet.

During the experimental period 3·45 g. of dihydrocholesterol, dissolved in butter, were taken, distributed through 12 meals. Immediately after defaecation the stools were ground with sodium sulphate and finely powdered, an accurately weighed sample (corresponding roughly to a 24-hour quantity) was extracted with ether and the amounts of total sterols and saturated sterols determined by Schönheimer's method. The precipitation of the digitonide of

the saturated sterols, employing 50 % excess of digitonin, was carried out after bromination in alcoholic solution. The solubility of the precipitates was determined, and the content of dihydrocholesterol calculated from curve 3, Fig. 1.

Table I. *Balance experiments with dihydrocholesterol.*

	Pre-experimental period	Experimental period 3·45 g. added dihydrocholesterol	1st post-experimental period	2nd post-experimental period
	g.	g.	g.	g.
Total unsaponifiable	5·35	9·91	7·22	5·71
Total sterol	4·23	8·42	5·88	4·26
Saturated sterol	3·65	7·41	5·20	3·42
Solubility of digitonides of saturated sterols in 2 ml. methyl alcohol:				
	mg.	mg.	mg.	mg.
Determination A	43·8	27·1	33·4	44·3
Determination B	43·9	27·6	35·0	43·6
Amount of dihydrocholesterol:				
Determination A	0 %	38 %	20 %	0 %
Absolute amount	0 g.	2·81 g.	1·04 g.	0 g.
Determination B	0 %	37 %	18 %	0 %
Absolute amount	0 g.	2·74 g.	0·94 g.	0 g.

From Table I it will be seen that dihydrocholesterol added to the diet was not excreted entirely during the experimental period, but a considerable portion appeared during the first post-experimental period. The whole amount of ingested dihydrocholesterol was recovered within these two periods. 3·85 g. dihydrocholesterol were found in the first determination and 3·68 g. in the second. The limit of error in the rather complicated experiment must however be considered relatively high, perhaps 10–20 %.

Under the influence of the relatively large quantities of ingested dihydrocholesterol the resorption of part of the cholesterol is apparently prevented, with resulting bacterial conversion into coprosterol (see the following paper), for during the pre-experimental period and the second post-experimental period 7·07 g. in all of saturated sterols were excreted. During the experimental and first post-experimental periods a total of 12·61 g. of saturated sterol was found, an increase of 5·54 g. Since 3·68–3·85 g. of this were found to consist of dihydrocholesterol, the missing 2 g. must have consisted of coprosterol. This cannot have been formed from the dihydrocholesterol inasmuch as the latter was recovered practically quantitatively.

The inhibition of the resorption of cholesterol by the ingestion of dihydrocholesterol can be explained on physical grounds, for cholesterol and dihydrocholesterol are known to form mixed crystals which cannot be separated by physical methods [Schönheimer, 1930; Lettré, 1932].

The amount of non-sterol unsaponifiable matter excreted in the experimental and first post-experimental periods is increased only to a small extent (10 %) over the corresponding excretion during the experimental and first post-experimental periods. This is barely beyond the limit of variation and thus supports the view that the non-sterol unsaponifiable matter does not originate from unresorbed cholesterol to more than a small extent.

SUMMARY.

A method is described for the determination of dihydrocholesterol in presence of coprosterol depending on the differential solubility of the corresponding digitonides in methyl alcohol.

With its aid it is shown that dihydrocholesterol added to the diet is not converted into coprosterol but reappears unchanged in the faeces.

' The author wishes to express his indebtedness to Prof. R. Schönheimer, formerly Head of the Department of Chemistry in the Pathological Institute, Freiburg, for many suggestions during the progress of this work.

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CXVI. THE FORMATION OF COPROSTEROL IN THE INTESTINE[1].

II. THE ACTION OF INTESTINAL BACTERIA ON CHOLESTEROL.

By HENRIK DAM.

From the Department of Chemistry, Institute of Pathology, Freiburg, Br., and the Biochemical Institute, University of Copenhagen.

(*Received April 28th, 1934.*)

Although various investigators [Bondzynski and Humnicki, 1896–97; Schönheimer *et al.*, 1930; Beumer and Bischoff, 1930] had reported the failure of attempts to convert cholesterol into coprosterol by bacteria *in vitro*, it was stated by Bischoff [1930, 1, 2] that the degree of saturation of faecal sterols increases when faeces are putrefied. This result, obtained by applying the iodine method of Bürger and Winterseel [1929] to sterols isolated from their digitonides by extraction with xylene, points to the probability that coprosterol can be formed by bacterial action.

An easier method is now available for the determination of total saturated sterols in the presence of unsaturated sterols [Schönheimer, 1930], and a method for the determination of dihydrocholesterol and coprosterol in a mixture has been described in the preceding paper. With the aid of these methods I have been able to confirm Bischoff's statement and to show that under the conditions of intestinal putrefaction cholesterol is converted not into dihydrocholesterol but into coprosterol.

Most of the putrefaction experiments were conducted quantitatively, and it was found that the total sterol was constant during putrefaction, indicating that intestinal bacteria are not able to destroy cholesterol or coprosterol.

Experimental.

The putrefaction experiments were generally carried out in an atmosphere of nitrogen or hydrogen although some were performed under aerobic conditions. When the putrefaction was conducted in the absence of oxygen, water or mercury seals were employed; under aerobic conditions the vessels were closed merely with a cotton plug. (In the aerobic experiments the processes in the inner mass of the faeces were most probably anaerobic because of the slow diffusion of gases from the surface.)

It was noted that in putrefaction under hydrogen the gas disappeared in amounts greatly exceeding those corresponding to the hydrogenation of the cholesterol.

The faeces were rubbed up to a uniform consistency in a mortar and weighed out with an accuracy of about 50 mg. Unless otherwise stated in the tables, 10 g. of faeces were employed in each experiment. The unsaponifiable matter

[1] This investigation was made possible by the assistance of the Josiah Macy, Jr., Foundation.

was isolated, and the saturated sterols were determined according to the method of Schönheimer [1930]. Only when the degree of saturation was very small (when cholesterol was added to the faeces or with a milk diet) did cholesterol dibromide crystallise from the alcohol on addition of bromine. In general 30–100 mg. of digitonide were obtained; only with low degrees of saturation were the amounts smaller than this, in which case the accuracy of the results was correspondingly influenced.

1. *Putrefaction of faeces without additions.*

Table I.

		Total sterols in 10 g.				
		Before incubation		After incubation		
	Diet	Amount mg.	Saturation %	Amount mg.	Saturation %	Incubation
1. Adult (H. D.)	Mixed	94·8	91·6	92	92·6	Aerobic, 49 hrs., 40°
2. ,, (S)	,,	63·0	78·6	65	88	In H_2, 7 days, 38°
3. ,, (corpse)*	,,	75·2	80·0	77·8	87·5	In H_2, 10 days, 38°
4. ,, (E. J.)	Milk†	59·8	69·6	61·8	79·5	In H_2, 48 hrs., 37°
5. ,, (I. D.)	Milk‡	85·2	2·5	85·3	1·8	In H_2, 67 hrs., 38·5°
6. Infant (8 days old)	Milk	81·2	8·5	80·2	9·1	Aerobic, 48 hrs., 37°

* Colon contents.
† 4 litres daily, beginning 4 days before experiment.
‡ 1 litre milk, 0·5 litre 30 % cream, 1 zwieback, daily, beginning 4 days before experiment.
p_H of the faeces = 8·1 (quinhydrone) (a few days earlier the faeces were acid).

In none of the experiments was a decrease observed in the total sterols during putrefaction, the difference lying within the limits of error. In Exps. 2, 3 and 4 hydrogenation of the sterols is clearly evident. No hydrogenation occurred in Exp. 1, which may be ascribed to the fact that the initial degree of saturation was very high (92 %). These experiments confirm the observation of Müller [1900] that the degree of saturation of the sterols falls on a milk diet. In cases of extremely low initial saturation (Exps. 5 and 6) no hydrogenation occurs during putrefaction.

2. *Putrefaction of the contents of the jejunum and ileum.*

The amount of cholesterol hydrogenated during putrefaction of colon contents and faeces even in the positive experiments could be only small, for the major part of the sterols existed at the outset in the form of coprosterol. An experiment (No. 7, not reported in the Tables) was accordingly undertaken with the united contents of the jejunum and ileum, in which hydrogenation has, as a rule, proceeded only to a slight extent. The material employed was obtained from the individual whose colon contents were examined in Exp. 3 (Table I). On analysis 10 g. of the fresh material showed 23·6 mg. of total sterols of which 2·5 % was saturated; after incubation for 10 days at 38° in H_2 the total sterols were 24·8 mg. with 2·6 % saturation. The hydrogenation of sterols thus appears to be associated with the contents of the large intestine.

3. *Putrefaction of faeces with added cholesterol.*

As the cholesterol for these experiments had to be in a form that could be attacked by bacteria, crystalline material was unsuitable. Egg-yolk, known to be rich in cholesterol, was employed; or cholesterol was either brought into

solution in bile acids and fatty acids or employed in the form of the water-soluble ester salts synthesised by Schönheimer and Breusch [1932]. In later experiments (Tables III and IV) colloidal cholesterol solutions secured from the firm of von Heyden were also employed. All added solutions were neutralised to litmus.

Table II.

		Substance	State	Total sterols in 10 g. Amount mg.	Total sterols in 10 g. Saturation %	Incubation
8.	Adult (H. D.)	35 g. faeces + 15 g. egg-yolk	Fresh	120	61·4	—
			Incub.	120	64·9	Aerobic, 48 hrs., 37°
9.	,, (E. J.)	100 g. faeces + 18 g. egg-yolk	Fresh	93·4	69·3	—
			Incub.	93·5	85·4	In H_2, 49 hrs., 39–43°
10.	,, (S)	8 g. faeces + 10 ml. sol. A	Fresh	—	33·2	—
			Incub.	—	36·1	In H_2, 4 hrs., 37°
			,,	—	34·4	In H_2, 47 hrs., 37°
11.	,, (corpse)	60 g. colon content + sol. B	Fresh	207	60·7	—
			Incub.	204·5	74·7	In H_2, 18 days, 37°
12.	,, (S)	8 g. faeces + 1·5 ml. sol. C	Fresh	115·2	43·8	—
			Incub.	114	50·8	In H_2, 7 days, 38°
			,,	117	52·3	In N_2, 7 days, 38°
			,,	113	43·8	Sterilised in boiling water-bath, then 7 days at 38° in H_2
13.	,, (S)	20 g. faeces + 3 ml. sol. D	Fresh	—	45	—
			Incub.	—	58·4	In H_2, 9 days, 37°

All were on mixed diet.
Solution A. 6·2 g. sodium desoxycholate, 1 g. sodium stearate and 0·35 g. cholesterol in 30 ml. water.
,, B. 1·2 g. sodium salt of monocholesterylbutanetetracarboxylic acid in 8 ml. water. The whole solution was added.
,, C. 1·5 g. sodium salt of monocholesterylbutanetetracarboxylic acid in 25 ml. water.
,, D. 0·6 g. sodium salt of monoallocholesterylbutanetetracarboxylic acid in 6·5 ml. water.

Cholesterol in egg-yolk or in the form of soluble preparations was only slightly hydrogenated, the increase in saturated sterols being little more than that obtained without the addition of cholesterol.

A parallel experiment in which allocholesterol was added in the form of its soluble acid ester (Exp. 13) was also carried out. The failure of an attempt to convert allocholesterol into coprosterol by putrefaction has been reported by Beumer and Bischoff [1930]; these authors however had heated the allocholesterol before the experiment, a procedure which may have converted it into cholesterol. The soluble allocholesterol ester was not converted any more rapidly than the analogous cholesterol ester. The addition of large quantities of bile acids and fatty acids appears to diminish the extent of hydrogenation.

4. *Effect of formates on putrefaction.*

Sodium formate, which is an efficient hydrogen donator for *B. coli* and other bacteria, was without influence.

In Exp. 14 the rather extensive hydrogenation was not increased by formate. Essentially the same result was obtained in Exp. 15, in which it was found that cholesterol betaine ester was practically not hydrogenated.

Table III.

	Substance	State	Saturation of sterols %	Incubation
14.	9 g. faeces + colloidal cholesterol solution	Fresh	62·7	—
		Incub.	96·5	In N_2, 6 days, 38°
		,,	92·5	Ditto, with 3 ml. 5 % H.COONa
15.	3 g. faeces + 86 mg. cholesterylbetaine ester hydrochloride	Fresh	22·9	—
		Incub.	23·5	Aerobic, 12 days, 38°
		,,	27·7	Ditto, with 3 ml. 5 % H.COONa

Subjects were adults on mixed diet.

5. *Attempted hydrogenation with pure bacterial cultures*[1].

Table IV.

		Saturation of sterols %
16.	Human bile (gall bladder), 170 ml. + 17 ml. 5 % H.COONa + 4·9 g. *B. coli*. Incubated aerobically for 10 days	4·0
17.	Brain hash (human) 62 g. + 6·2 ml. 5 % H.COONa inoculated with *B. coli*. Incubated *in vacuo* 5 days	1·9
18.	Brain hash (human) 37 g. + 3·6 ml. 5 % H.COONa, inoculated with *B. coli*. Incubated *in vacuo* 5 days	0·3
19.	Colloidal cholesterol in protein (von Heyden) 1 g. + 4 ml. water + 700 mg. *B. coli*. Incubated *in vacuo* 12 days	5·7
20.	Colloidal cholesterol in protein, 1 g. + 5 ml. 5 % H.COONa + 1·16 g. *B. coli*. Incubated *in vacuo* 12 days	0·6
21.	Colloidal cholesterol solution (von Heyden), 5 ml. + 20 ml. nutrient bouillon with 10 % glucose, inoculated with *Enterococcus* Type B. Incubated aerobically 2 days	*

* The amount was too small to weigh.

The action of pure bacterial cultures was, in spite of the large amounts of bacteria employed, barely perceptible, and certainly considerably less than in putrefaction of faeces.

6. *Nature of the saturated sterols formed during putrefaction.*

The digitonin-precipitable, saturated sterols formed from cholesterol can consist only of coprosterol or dihydrocholesterol, so that the isolation of the saturated sterol formed by putrefaction cannot be accomplished by methods at present available. In the previous communication however a method has been described for determining which of the two sterols has been formed. Since relatively large quantities of sterol are necessary for the solubility determinations, the digitonides from Exps. 2 and 12 were employed. Both experiments involved the same sample of stool, of which a large quantity was available. In Exp. 12 soluble cholesterol ester was added. The appropriate combined digitonides from Exps. 2 and 12 were united for the determination of sterols before and after putrefaction, respectively. During putrefaction 11–13 % of saturated sterols had formed; if these had consisted of dihydrocholesterol, the solubility of the digitonides in methyl alcohol would have been considerably lower than before putrefaction, whereas if coprosterol had formed, the solubility would barely have changed. Actually the solubilities were 42·0 and 43·4 mg. in 2 ml.

[1] Thanks are due to Miss R. Marget for valuable help in the bacteriological work.

of methyl alcohol respectively, before and after putrefaction. If dihydrocholesterol had been the sole product of hydrogenation, the solubility of the sterol-digitonide after putrefaction would have been about 37 mg.

Discussion.

The observation that cholesterol and coprosterol are not degraded by intestinal bacteria is of importance in the interpretation of cholesterol balance experiments. It is therefore possible to ascribe the destruction of cholesterol in the body [Dam, 1931; Page and Menschick, 1932; Menschick and Page, 1933; Schönheimer and Breusch, 1933] to a process of intermediary metabolism rather than to the agency of intestinal bacteria.

The present results confirm those of Bischoff [1930, 1, 2] in indicating that the amount of saturated sterols increases, but they differ from them quantitatively. In one case Bischoff found an increase from 12 to 66 %; I have never observed so large a change. The values found by Bischoff for the degree of saturation of the sterols of normal human faeces are rather low in comparison with the observations of Schönheimer and von Behring [1930] and those here reported which were found by the gravimetric method of Schönheimer. This discrepancy may perhaps be explained by some technical error in the determinations of Bischoff—an interpretation supported by the statement of Bischoff that a coprosterol with a melting-point of 97° employed by him was precipitable by digitonin only to the extent of 60 %.

Although hydrogenation took place only to a small extent on putrefaction, its occurrence was conclusively established in almost every experiment with normal faeces. It failed to occur only when the initial degree of saturation was extremely high (Exp. 1), and in some of the milk diet experiments in which the initial saturation was extremely low. Complete hydrogenation of unsaturated sterol was never attained, even on long incubation.

Added cholesterol, whether in the form of egg-yolk, as soluble ester or in colloidal solution, remained largely unattacked by the bacteria. It thus seems possible that the cholesterol exists in the colon in some unknown form which is especially susceptible to bacterial action.

The hydrogenation is certainly a bacterial process. It does not take place after sterilisation (Exp. 12), and it is associated particularly with the contents of the colon, for it takes place neither in the small intestine nor on the inoculation of cholesterol-containing organs or soluble cholesterol preparations with bacterial cultures. It may be supposed that either the optimum conditions for the growth of the appropriate bacteria can be obtained only in the colonic secretions, or these secretions contain special hydrogen donators. Sodium formate can apparently not be employed by the bacteria for the formation of coprosterol. Better results might perhaps be obtained by the use of sterile colon excretions as nutrient medium, but such material was not available.

The identity of the causative bacteria could not be determined.

The proof, by solubility determinations of the digitonides, that the hydrogenated sterol is not dihydrocholesterol but coprosterol establishes the fact, long suspected, that it is coprosterol which is formed by bacterial hydrogenation of cholesterol.

Summary.

In the putrefaction of faeces the degree of saturation of the sterols rises owing to formation of coprosterol from cholesterol. The total amount of sterol remains constant.

The author wishes to acknowledge his indebtedness to Prof. R. Schönheimer, formerly Head of the Department of Chemistry, Institute of Pathology, Freiburg, for many helpful suggestions.

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CXVII. THE MELTING-POINT AS A TEST FOR THE PURITY OF COPROSTEROL[1].

By HENRIK DAM.

From the Department of Chemistry, Pathological Institute, University, Freiburg, Br.

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Recent work on the metabolism of saturated sterols makes it necessary to know the reliability of the usual tests for the purity of coprosterol.

The degree of saturation can be easily determined by the bromine-digitonin method of Schönheimer [1930] with an accuracy of about 1 %, so that it is possible to detect cholesterol as an impurity in the preparation. This method can also be used for the removal of any cholesterol, thus rendering it possible to obtain a preparation with a saturation of nearly 100 %, even in cases where the crude product contains a large amount of unsaturated sterols.

As shown by Windaus and Uibrig [1915], Gardner [1921] and Schönheimer [1930], dihydrocholesterol occurs in the sterol fraction of faeces and may therefore be found as an impurity in coprosterol prepared from this source.

The specific rotations for coprosterol and dihydrocholesterol are $[\alpha]_D + 23{\cdot}55°$ [Windaus, 1916] and $+28{\cdot}8°$ [Willstätter and Mayer, 1908] respectively, and are thus too close for use in the simultaneous determination of these two sterols.

The melting-point of coprosterol is variously given as 95–96° [Bondzynski and Humnicki, 1896–97], 100° [Dorée, 1913], 112–116° [Fischer, 1911], 101–102° [Windaus, 1916]; a product prepared from pseudocoprosterol melted at 105–107° [Uibrig, 1915]. The highest recorded (Fischer) was on a sample many times recrystallised, but subsequently [Windaus and Uibrig, 1915] shown nevertheless to contain dihydrocholesterol. An equally high melting-point has also been reported by Schönheimer [1929] and by the present author [1925].

In order to ascertain the depression of the melting-point of coprosterol by either dihydrocholesterol or cholesterol, the melting-point diagram has been determined by the method of Rheinboldt and Kircheisen [1926] and Lettré [1932].

The material used was prepared from human faeces collected on a diet (white bread, butter, meat, eggs, potatoes and barley porridge) poor in plant sterols. The digitonide of the unsaponifiable fraction was decomposed by extraction with xylene and the sterol recrystallised from methyl alcohol. It melted at 100–101°, unchanged after repeated crystallisation. The degree of saturation (method of Schönheimer) was 98·7 %. Only a faint yellow colour was obtained in the Salkowski reaction.

The melting-point diagram shows that the presence of up to about 50 % of dihydrocholesterol will produce only a slight depression of the melting-point of the coprosterol. The softening-points of the mixtures were materially lower, but very difficult to determine.

[1] This investigation was made possible by a grant from the Josiah Macy, Jr., Foundation.

Fig. 1 shows that in the neighbourhood of 100° the sharpness of the melting-point is of much greater importance as a test for purity than the melting-point itself. A melting-point of 110–115° represents a mixture with more than 60 % dihydrocholesterol.

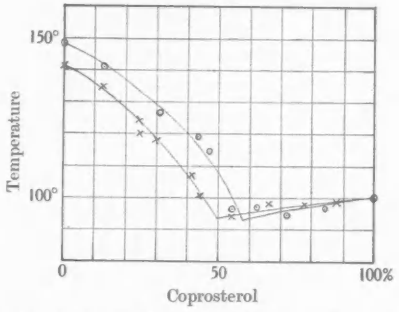

Fig. 1. ×—× Dihydrocholesterol-coprosterol; ⊙—⊙ Cholesterol-coprosterol.

It is a common experience that crude coprosterol preparations, even when extracted with xylene from digitonides precipitated in presence of bromine, show a melting-point of about 80°, thus indicating that the substances in question are not simple mixtures of coprosterol and dihydrocholesterol.

Summary.

The tests for purity of coprosterol have been discussed in connection with melting-point diagrams for coprosterol-dihydrocholesterol and coprosterol-cholesterol.

The author wishes to acknowledge his indebtedness to Prof. R. Schönheimer, formerly Head of the Department of Chemistry in the Pathological Institute, Freiburg, for many suggestions during the progress of this work.

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CXVIII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXXVIII. THE METABOLIC PRODUCTS OF *PENICILLIUM MINIO-LUTEUM* DIERCKX. MINIOLUTEIC ACID.

By JOHN HOWARD BIRKINSHAW and HAROLD RAISTRICK.

From the Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London.

(*Received April 25th, 1934.*)

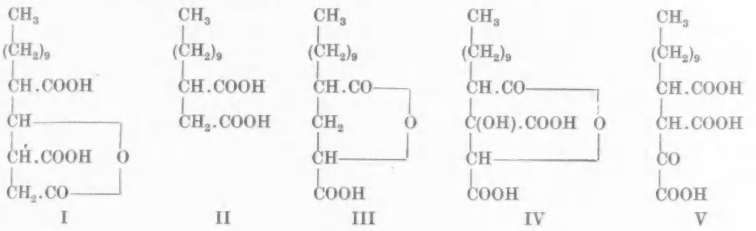
Penicillium minio-luteum Dierckx was described by R. P. Dierckx [1901] and is referred to by Biourge [1923, p. 237] and by Thom [1930, p. 464] in their monographs on the genus *Penicillium*. We have found that when this organism is grown at 24° on Czapek-Dox medium containing glucose as the sole source of carbon a highly pigmented metabolism solution is formed which froths very badly on vacuum-evaporation. The metabolism solution gives heavy amorphous precipitates with aqueous solutions of the salts of many heavy metals, *e.g.* barium, calcium, copper, nickel, lead, iron, and with silver on standing, but gives no precipitate with mercuric salts. Further, on strongly acidifying the metabolism solution a somewhat pigmented crystalline precipitate is formed on standing. Investigation of the barium and copper precipitates and also of the crystalline precipitate formed on acidification has led to the isolation of two colourless crystalline acids: (1) The γ-lactone of γ-hydroxy-$\beta\delta$-dicarboxypentadecanoic acid, $C_{17}H_{28}O_6$ (I). This acid was previously described by Clutterbuck *et al.* [1931] as a metabolic product of *P. spiculisporum* Lehman, and is now for convenience given the name *spiculisporic acid*. (2) A hitherto undescribed, dextrorotatory acid having the empirical formula $C_{16}H_{26}O_7$, for which the name *minioluteic acid* is proposed. It is interesting to note that Thom [1930] places *P. spiculisporum* Lehman and *P. minio-luteum* Dierckx in the *Biverticillata-Symmetrica* group of species of *Penicillium* on morphological grounds.

Minioluteic acid titrates in the cold as a dibasic acid, but a third acidic group is unmasked by heating with an excess of aqueous alkali. Since the third carboxyl group is masked again on acidification, minioluteic acid is obviously a lactonic acid. This view is supported by the fact that minioluteic acid gives a dimethyl ester and a trihydrazide. Since minioluteic acid contains no unsaturated linkages, and apart from the lactone ring must, from its empirical formula, be an open chain compound, it is obviously the monolactone of a dihydroxytricarboxylic acid.

On fusion with potash, oxalic acid, *n*-dodecanoic acid and a dibasic acid having the empirical formula $C_{14}H_{26}O_4$ were obtained. This acid was proved by synthesis to be decylsuccinic acid (II).

On reduction with sodium amalgam, minioluteic acid gives the lactone of a dicarboxylic acid, $C_{15}H_{26}O_4$, which has been shown by synthesis to be the γ-lactone of α-hydroxy-γ-carboxy-*n*-tetradecanoic acid (III).

On the basis of these facts it appears that minioluteic acid is the γ-lactone of $\alpha\beta$-dihydroxy-$\beta\gamma$-dicarboxy-*n*-tetradecanoic acid (IV).



Experimental.

Organism.

The organism used in the whole of this work was a culture of *P. minio-luteum* Dierckx (London School of Hygiene and Tropical Medicine Catalogue No. P 44), received originally through the courtesy of Prof. P. Biourge of the University of Louvain in December 1929. It bears Prof. Biourge's Catalogue No. 60, and is actually a subculture of the original organism isolated by Dierckx.

Culture medium and course of metabolism.

The medium used was the ordinary Czapek-Dox solution of the following composition: glucose, 50 g.; $NaNO_3$, 2 g.; KH_2PO_4, 1 g.; KCl, 0·5 g.; $MgSO_4$, $7H_2O$, 0·5 g.; $FeSO_4$, $7H_2O$, 0·01 g.; water to 1000 ml.

Since preliminary experiments showed that better growth and quicker metabolism were obtained at 24° than at 30° the former was used throughout as the temperature of incubation. Batches of 100 one-litre conical flasks, each containing 350 ml. of the above Czapek-Dox solution, were sterilised and sown with a spore suspension of *P. minio-luteum* and incubated at 24°. Growth was fairly rapid at first and produced a whitish mycelium with green areas of conidial growth. As growth proceeded the mycelium became predominantly green with pinkish patches. The metabolism solution, which quickly developed a faint purple colour, gradually became darker in colour until after 5 weeks it was very dark brown. At the end of the incubation period the contents of the flasks in each batch were filtered and treated by one or other of the following methods:

Method A. This method depends on the fact that both minioluteic and spiculisporic acids are precipitated from aqueous solution by various metallic salts. The filtered metabolism solution and washings, having p_H 4·2–4·3, were treated with a 40 % aqueous solution of barium acetate (128 g.). The precipitate was filtered off and to the filtrate, which now had p_H 5·2, 100 g. of copper acetate dissolved in about 1 litre of hot water were added. The filtrate from the copper precipitate, having p_H 5·0, was treated with a slight excess of aqueous normal lead acetate solution, and the filtrate from this precipitate was brought to p_H 6·2 by the addition of 450 ml. 2*N* NaOH and precipitated with basic lead acetate solution. The acids contained in the various precipitates were now extracted as follows:

1. *Barium precipitate.* The barium precipitate was ground with an excess of an aqueous solution of sodium sulphate at 60° and filtered hot; the filtrate was acidified with a slight excess of 2*N* H_2SO_4 and extracted twice with rather less than its own volume of ether. On evaporation of the ether a semicrystalline

solid, weight 46·1 g., remained. On fractional crystallisation from water and decoloration with norite a less soluble fraction, weight 17·3 g., of almost pure minioluteic acid, melting at 170° was obtained. The more soluble fraction was fractionated from a mixture of ether and light petroleum giving 3·7 g. of somewhat impure spiculisporic acid, which on further purification melted at 142–144°. This was identified by comparison with an authentic specimen of spiculisporic acid from *P. spiculisporum*.

2. *Copper precipitate.* The copper precipitate was ground with water, treated with 150 ml. 5*N* HCl in which practically the whole dissolved, and extracted with ether. The ether extract on evaporation gave 37·9 g. of a dark-coloured semi-crystalline mass, from which by fractionation from hot water 17·7 g. of crystalline material were obtained. Nothing but spiculisporic acid (M.P. 144–145·5°) could be isolated from this fraction. No minioluteic acid could be isolated from the copper precipitate.

3. *Lead precipitates.* The normal lead and basic lead precipitates were separately ground with excess of dilute sulphuric acid and filtered from the precipitated lead sulphate, and the filtrate was extracted with ether. The ether extract from the normal lead acetate precipitate proved to be an uncrystallisable syrup from which nothing was isolated. The ether extract from the basic lead acetate precipitate consisted essentially of an uncrystallisable syrup. A small amount (0·6 g.) of a complex mixture of colourless crystalline substances was however isolated, but this has not been further investigated.

Method B. The combined filtered metabolism solution and washings from one batch of 100 flasks were acidified with 3 litres of concentrated HCl and left in the cold room overnight. The precipitated pigmented, crystalline material was filtered off, washed, dried and weighed (80·5 g.). It consisted of a mixture of minioluteic, spiculisporic and possibly other acids, which up to the present have not been isolated in a state of purity. The crude pigmented crystalline mixture was recrystallised six times from hot water (norite), giving 40 g. of colourless material, M.P. 150–156°. Minioluteic acid was separated from this material by taking advantage of the fact that, in contrast with spiculisporic acid, it forms a potassium salt which is insoluble in ethyl alcohol. 40 g. were dissolved in 1 litre of ethyl alcohol, and a solution containing 24 g. of anhydrous potassium acetate in 500 ml. of ethyl alcohol was added slowly and with constant shaking. The precipitated potassium salt was filtered off after half an hour, washed with ethyl alcohol, dissolved in about 900 ml. of warm water and treated with 90 ml. of concentrated HCl. After cooling, the acid was filtered off and dried; yield 34 g., M.P. 164–167°. The alcoholic mother-liquors were evaporated

Table I.

Exp. No.	Incubation period in days	Apparent residual glucose by polarimeter %	Final p_H	Method of isolation	Yields of crude products g.	
1	47	0·23	4·3	A	*Ex* Ba precipitate	46·1
					Ex Cu precipitate	37·9
					Ex basic Pb precipitate	9·0
2	52	0·11	4·2	A	*Ex* Ba precipitate	52·5
					Ex Cu precipitate	37·1
					Ex normal Pb precipitate	4·7
					Ex basic Pb precipitate	13·0
3	50	0·10	—	B		80·5
4	53	0·16	—	B		75·5

in vacuo and dissolved in water, and the acid was precipitated by HCl; yield 3·3 g., M.P. 134–137°. The main fraction melting at 164–167° was now re-precipitated as the potassium salt which was further purified by crystallising once from water. By this means 18·3 g. of pure minioluteic acid melting at 171° were isolated. Pure spiculisporic acid was obtained by fractional crystallisation of the material from the alcohol-soluble potassium salt.

The experimental details of each of four batches of 100 flasks each are given in Table I.

General properties of minioluteic acid.

Minioluteic acid is best purified by crystallisation from hot water from which solvent it separates in colourless needles melting at 171°. (Found by Schoeller: C, 58·00, 57·96; H, 7·65, 7·72 %; OCH_3, nil; N, nil; mol. wt., 317, 332. $C_{16}H_{26}O_7$ requires: C, 58·14; H, 7·93 %. Mol. wt., 330.)

Titration. 0·1576 g. dried to constant weight at 105° and dissolved in 10 ml. ethyl alcohol required 9·55 ml. *N*/10 NaOH corresponding to an equivalent of 165 ($C_{16}H_{26}O_7$ titrating as a dibasic acid requires 165). A further 20·00 ml. *N*/10 NaOH were now added, the mixture heated on the water-bath for 2 hours, cooled and back-titrated with *N*/10 HCl. A further 4·68 ml. *N*/10 NaOH were neutralised during hydrolysis, making a total of 14·23 ml. *N*/10 NaOH and corresponding with an equivalent of 110·7 ($C_{16}H_{26}O_7$ titrating as a tribasic acid requires 110). Minioluteic acid thus titrates in the cold as a dibasic, and on heating as a tribasic acid. 14·23 ml. *N*/10 HCl were now added and the mixture allowed to stand at room temperature for 24 hours. On back-titration 9·48 ml. *N*/10 NaOH were required. These figures show that reversion of the tribasic form to the dibasic form was complete and indicate the probable presence of a γ-lactone ring.

The above conclusions were confirmed by the preparation of the silver salts of the open and closed ring forms which were found to contain 47·80 and 39·35 % Ag respectively. (Theoretical for $C_{16}H_{25}O_8Ag_3$, 48·39 %, and for $C_{16}H_{24}O_7Ag_2$, 39·67 %.)

It was found impossible to isolate the open ring form of minioluteic acid in the free state. A quantity of minioluteic acid was hydrolysed by heating with a measured excess of *N* NaOH. The cooled hydrolysate was shaken with ether and rather less than the theoretical amount of *N*/10 HCl cautiously added with constant shaking, 1–2 ml. at a time. The ethereal solution was treated at room temperature with light petroleum and the material separating was air-dried. It showed an equivalent on titration of 169. (Theoretical for closed form 165, for open form 116.) In a further attempt to prepare the trimethyl ester of the open form of minioluteic acid the ethereal solution as obtained above was methylated directly with diazomethane. The resultant crystalline product proved to be the dimethyl ester, M.P. 86° (see p. 832).

Rotations. (*a*) Free acid in acetone

$$c=1\cdot07.\ [\alpha]^{16}_{5461}+108\cdot1°,\ [\alpha]^{16}_{5790}+94\cdot5°.$$

(*b*) Disodium salt in water, calculated as the free acid.

$$c=1\cdot04.\ [\alpha]^{19}_{5461}+62\cdot4°,\ [\alpha]^{19}_{5790}+54\cdot6°.$$

Solubilities. Minioluteic acid is readily soluble in cold ether, ethyl alcohol, acetone, ethyl acetate, butyl alcohol and hot water. It is somewhat soluble in chloroform (1:400) and is practically insoluble in cold water, benzene and light petroleum.

Salts. Minioluteic acid gives insoluble salts with a large number of metals and in this respect is very similar to spiculisporic acid. A 1 % neutral solution of the sodium salt of minioluteic acid gave heavy precipitates with silver nitrate, ferric chloride, lead acetate and the chlorides of calcium, barium, nickel and zinc; moderate precipitates with copper sulphate and magnesium chloride, and no precipitate with mercuric chloride, uranium acetate and aqueous potassium chloride. In contrast with spiculisporic acid, minioluteic acid gives a potassium salt which is almost insoluble in alcohol, the potassium salt of spiculisporic acid being relatively soluble in this solvent.

Dimethyl ester. Minioluteic acid, dissolved in ether, was treated with an excess of an ethereal solution of diazomethane. Vigorous evolution of nitrogen took place and the ethereal solution was filtered, evaporated and the crystalline residue recrystallised from a mixture of chloroform and light petroleum. Colourless plates, M.P. 86·5°. (Found: C, 60·61, 60·58; H, 8·44, 8·22; OCH_3, 17·41, 17·64 %. $C_{16}H_{24}O_5(OCH_3)_2$ requires C, 60·30; H, 8·44; OCH_3, 17·32 %.)

Trihydrazide. 0·08 g. of the dimethyl ester of minioluteic acid was dissolved in 2–3 ml. ethyl alcohol and 0·16 ml. of 50 % aqueous hydrazine hydrate added. Deposition of micro-crystals began immediately. These were drained, washed with ethyl alcohol and dried *in vacuo* over H_2SO_4. As no suitable crystallising medium could be found the compound was analysed without recrystallisation. The crystals sintered with incipient decomposition at 157–160° and melted finally at 201°. (Found: C, 49·37, 49·22; H, 8·72, 8·60; N, 21·30, 21·30 %. $C_{16}H_{34}O_5N_6$ requires C, 49·19; H, 8·78; N, 21·52 %.)

A saturated aqueous solution of minioluteic acid does not reduce acid potassium permanganate in the cold although the permanganate is slowly reduced on heating. An alcoholic solution of the acid showed no absorption of hydrogen after 45 minutes' shaking with palladised norite. With Wijs's iodine solution 1 g.-mol. of minioluteic acid absorbed 0·845 g.-atom of iodine after 2 hours, but this is almost certainly due to chemical reactions other than the simple addition of iodine. These facts together with the analytical figures obtained for minioluteic acid and its dimethyl ester and trihydrazide indicate the absence from the molecule of the acid of an unsaturated linkage.

Potash fusion of minioluteic acid.

1 g. minioluteic acid was warmed with 5 g. solid KOH and 3 ml. water in a nickel crucible. When dissolution was complete the temperature of the metal-bath was raised slowly to 310° and maintained at this temperature for one hour. The melt was dissolved in water and acidified with dilute H_2SO_4. There was a considerable evolution of CO_2 and a slight smell of butyric acid. The mixture was kept in the cold room overnight and filtered from precipitated material, and the filtrate and precipitate were dealt with separately.

(*a*) *Filtrate.* The filtrate was exhaustively extracted with ether in a continuous extraction apparatus for 30 hours. On evaporation of the ether a crystalline residue, weight 0·41 g., was obtained. This was dissolved in water, titrated with *N* NaOH (equivalent = 61), the neutralised solution was acidified with acetic acid and the oxalic acid removed by precipitation with calcium chloride. Weight of calcium oxalate = 0·50 g. The oxalic acid-free solution was now acidified with H_2SO_4 and re-extracted with ether. The ether solution on evaporation left only a trace of residue from which no succinic or other crystalline acid could be isolated.

(*b*) *Precipitate.* Weight 0·48 g. This was purified by crystallisation from a mixture of ether and chilled light petroleum giving a small quantity (0·06 g.)

of colourless, crystalline plates, M.P. 87–89°, which on recrystallisation from acetone and water melted at 90–91° and had a barely detectable optical rotation. (Found: C, 65·02, 64·85; H, 9·99, 10·12 %. Mol. wt., 246, 250. $C_{14}H_{26}O_4$ requires: C, 65·07; H, 10·16 %; mol. wt., 258.) Equivalent by titration 130. (Theoretical for $C_{14}H_{26}O_4$ titrating as a dibasic acid, 129·1.) This acid was subsequently shown by synthesis to be decylsuccinic acid (II).

It was evident that a second acid was present in the precipitate (*b*). Hence the combined precipitates (*b*) from the potash fusion of 5 g. miniloluteic acid were distilled *in vacuo* at 16 mm. with the following results:

Fraction I, collected up to 135°. Weight 0·45 g. Softens at 30°. M.P. 38–40°.

Fraction II, collected from 135 to 145°. Weight 0·57 g. Softens at 34°. M.P. 44–44·5°.

Fraction III. B.P. gradually fell to 100°. Weight 0·39 g. Softens at 41°. M.P. 43–48·5°.

Fraction IV. The residue in the distillation-flask was now distilled at 7 mm. when a fourth fraction distilled at 182°. Weight 0·74 g. M.P. about 58°.

Fraction I was esterified with diazomethane in ether and the residue on evaporation of the ether was treated with 2·7 ml. ethyl alcohol and 1 ml. 50 % aqueous hydrazine hydrate and allowed to stand for 24 hours. A total yield of 0·32 g. of hydrazide was obtained, which was crystallised twice from aqueous alcohol and once from benzene, M.P. 104–105°. A sample of *n*-dodecanoic acid hydrazide prepared similarly melted at 106–107°, and there was no depression in melting-point on mixing the two samples. (Found: C, 67·25, 67·34; H, 11·82, 11·98; N, 13·41, 13·25 %. *n*-Dodecanoic acid hydrazide, $C_{12}H_{26}ON_2$ requires: C, 67·23; H, 12·24; N, 13·07 %.)

Fraction IV, distilling at 182°/7 mm., was recrystallised several times from ether-light petroleum giving finally colourless needles, which after sublimation had M.P. 66–67°. This was shown to be the anhydride of decylsuccinic acid (see below).

Synthesis of decylsuccinic acid (II).

The method adopted was to condense ethyl-α-bromo-*n*-dodecanoate with ethyl sodiomalonate, and then to decarboxylate the tricarboxylic acid obtained on hydrolysis.

*α-Bromo-*n*-dodecyl bromide and ethyl α-bromo-*n*-dodecanoate.* α-Bromo-*n*-dodecyl bromide was synthesised by the method of Auwers and Bernhardi [1891]. *n*-Dodecanoic acid (10 g.) and red phosphorus (0·52 g.) were placed in a flask fitted with a reflux condenser and bromine (14·7 g.) added slowly. The temperature was slowly raised to 90–100°. A little more bromine was added at intervals until the evolution of HBr ceased. The mixture was cooled and the crude product converted, without previous isolation, into ethyl α-bromo-*n*-dodecanoate by the addition of ethyl alcohol (30 ml.). The mixture was kept for some hours, the excess of alcohol removed *in vacuo*, the residue dissolved in ether, washed with water and aqueous sodium carbonate solution, dried over anhydrous Na_2SO_4 and distilled *in vacuo*. The main fraction (13·7 g.) distilled at 115–123°/1 mm.

Triethyl αβ-dicarboxytridecanoate. Sodium (0·88 g.) was dissolved in ethyl alcohol (12 ml.) and ethyl malonate (6·1 g.) added. After half an hour ethyl α-bromo-*n*-dodecanoate (11·7 g.) was added and the mixture heated for 2 hours on the water-bath under reflux. Excess ethyl alcohol was removed by vacuum evaporation, water was added to dissolve the sodium bromide and the mixture was extracted with ether, the extract being dried with Na_2SO_4 and fractionated *in vacuo*. The main fraction (11·5 g.) distilled at 172°/1 mm.

αβ-Dicarboxytridecanoic acid. It was found that the triethyl ester was difficult to hydrolyse completely with alcoholic potash, but satisfactory results were obtained by fusion with potash. The ester was heated with five times its weight of stick potash and three times its weight of water, the temperature of the bath being raised as rapidly as frothing would permit to 300°. The melt was cooled, dissolved in water, acidified, and the beautifully crystalline product filtered off

(M.P. 148°, decomp.). It was recrystallised from a mixture of ether and four volumes of light petroleum. Colourless needles, M.P. 148° (decomp.). (Found: C, 59·27, 59·37; H, 8·56, 8·60 %. $C_{15}H_{26}O_6$ requires: C, 59·57; H, 8·67 %. Equivalent by titration, 100·4. Theoretical for $C_{15}H_{26}O_6$ titrating as a tribasic acid, 100·7.)

Decylsuccinic anhydride. αβ-Dicarboxytridecanoic acid was decarboxylated by distillation *in vacuo*. The product distilled at 160°/2 mm. and solidified in the receiver. It separated from light petroleum in colourless plates, M.P. 65–66°; after sublimation 68·5°. When mixed with the anhydride, M.P. 66–67°, obtained from minioluteic acid (Fraction IV, p. 833) it showed no depression in melting-point. (Found: C, 69·90; H, 9·88 %. $C_{14}H_{24}O_3$ requires: C, 69·93; H, 10·07 %.)

Decylsuccinic acid. Decylsuccinic anhydride (0·50 g.) was heated with 10 ml. *N* NaOH for half an hour on the water-bath. A back-titration with *N* HCl gave an apparent equivalent of 116 (theoretical 120). Sufficient excess of HCl was added to precipitate all the acid. The precipitate (0·5 g.) was recrystallised first from light petroleum and then from aqueous acetone, forming colourless glistening plates, M.P. 90–91°. This melting-point showed no depression on mixing with the dibasic acid, M.P. 90–91°, obtained by potash fusion of minioluteic acid (see p. 833).

Reduction of minioluteic acid with sodium amalgam.

Minioluteic acid (2 g.) was dissolved in 2 *N* NaOH (25 ml.) and water (75 ml.) added. Sodium amalgam (100 g., 2·5 % sodium) was added and the mixture heated on the water-bath. After 4 hours a further 100 g. sodium amalgam were added and so on until a total of 500 g. had been added. From time to time a little concentrated HCl was added to reduce the alkalinity, but the solution was never allowed to become acid. Finally, the mixture was cooled, acidified with HCl, extracted with ether and the ether removed, giving a residue, weight 1·37 g. The residue appeared to be a mixture of substances and was ground and extracted in a Soxhlet with light petroleum, B.P. 60–80°, giving 0·1 g. of a colourless crystalline product, M.P. 114°. The sticky residue which was now difficult to extract was dissolved in absolute alcohol and treated with an alcoholic solution of potassium acetate, giving a precipitate from which no crystalline material could be separated. The alcoholic solution was evaporated *in vacuo* and extracted with cold water. The insoluble portion was dissolved in hot water and acidified. The resultant precipitate, weight 0·16 g., M.P. 113°, was identical with the acid previously extracted with light petroleum, M.P. 114°. The combined fractions were recrystallised from ether-light petroleum giving colourless plates, M.P. 114·5°, which, after sublimation in a high vacuum, was raised to 119°. (Found: C, 66·82, 66·92; H, 9·37, 9·32 %. $C_{15}H_{26}O_4$ requires: C, 66·61; H, 9·70 %.)

0·0856 g. required 3·15 ml. *N*/10 NaOH for neutralisation in the cold, and on heating with excess *N*/10 NaOH required a further 3·37 ml. corresponding to equivalents of 272 and 131. $C_{15}H_{26}O_4$ titrating as a mono- or di-basic acid requires 270 or 135 respectively.

This reduction product, $C_{15}H_{26}O_4$, which is obviously the monolactone of a dibasic acid, thus differs from minioluteic acid, $C_{16}H_{26}O_7$, by one atom of oxygen and one molecule of CO_2. It has been shown by synthesis to be the γ-lactone of α-hydroxy-γ-carboxy-*n*-tetradecanoic acid (III).

*Synthesis of the γ-lactone of α-hydroxy-γ-carboxy-*n*-tetradecanoic acid (III).*

Ethyl decylsuccinate was condensed with ethyl oxalate in the presence of sodium ethoxide in ether, according to the method of Blaise and Gault [1911]. The resulting condensation product was hydrolysed and decarboxylated in alkaline solution and subsequently reduced with sodium amalgam.

Diethyl decylsuccinate. Decylsuccinic acid (16 g.) was esterified in the usual manner with two successive amounts of absolute ethyl alcohol (100 ml.) containing 3 % of HCl. The resultant ester was distilled *in vacuo*, B.P. 155–159°/2 mm., weight 15·9 g.

*α-Keto-γ-carboxy-*n*-tetradecanoic acid.* Powdered metallic sodium (1·15 g.) was treated with ethyl alcohol (2·3 g.) diluted with 20–30 ml. of ether. The mixture was allowed to stand overnight and ethyl oxalate (7·3 g.) added. After 2 hours ethyl decylsuccinate (15·7 g.) was added and the mixture left at room temperature for 48 hours. The mixture was poured into 20 ml. of 20 % H_2SO_4 and 25 g. of ice, shaken and the ether layer separated. In the method described by Blaise and Gault [1911] for the preparation of α-keto-γ-methylglutaric acid, on which this method is based, the corresponding triethyl ester was extracted from the ether solution at this stage by means of aqueous (25 %) potassium carbonate. A similar procedure in our hands failed to extract any triethyl α-keto-βγ-dicarboxy-*n*-tetradecanoate, probably owing to the feebler acidity of our ester. The ether solution was therefore extracted with *N* NaOH. By this means 6·7 g. of material were extracted whilst 11·7 g. of presumably uncondensed esters remained in the ether. The NaOH solution was now heated at 100° for 14 hours, cooled, acidified and extracted with ether. The ether solution was evaporated, giving a semicrystalline residue (4·2 g.), from which the liquid portion and a quantity of decylsuccinic acid were separated by fractionation from ether-light petroleum. The remainder (1·2 g.) consisted of impure α-keto-γ-carboxy-*n*-tetradecanoic acid. A pure sample of this acid was not prepared, but it was characterised by the preparation of its 2:4-dinitrophenylhydrazone.

*2:4-Dinitrophenylhydrazone of α-keto-γ-carboxy-*n*-tetradecanoic acid.* This was prepared by Brady's method [1931] from the crude product referred to above and was recrystallised from 70 % aqueous ethyl alcohol. Yellow needles, M.P. 180°. (Found on material dried to constant weight at 100°: C, 54·40; H, 6·62; N, 11·90 %. $C_{21}H_{30}O_8N_4$ requires: C, 54·05; H, 6·49; N, 12·01 %.)

*γ-Lactone of α-hydroxy-γ-carboxy-*n*-tetradecanoic acid (III).* The crude acid (0·7 g.) was reduced in alkaline solution with sodium amalgam (400 g., 2·5 % sodium) over a period of 16 hours on a boiling water-bath. The method used was similar to that followed for the hydrolysis and reduction of minioluteic acid. The resultant product was purified by fractionation from ether-light petroleum. Colourless plates. Weight 0·3 g. M.P. 119°, alone or in admixture with the lactone obtained by hydrolysis and reduction of minioluteic acid (p. 834). (Found: C, 66·37; H, 9·66 %. $C_{15}H_{26}O_4$ requires: C, 66·61; H, 9·70 %.)

Summary and discussion.

The metabolic products of *P. minio-luteum* Dierckx, when grown at 24° on Czapek-Dox medium containing glucose as the sole source of carbon, have been investigated. Two aliphatic acids of complex structure have been isolated: (*a*) the γ-lactone of γ-hydroxy-βδ-dicarboxypentadecanoic acid (I) previously described by Clutterbuck *et al.* [1931] as a metabolic product of *P. spiculisporum* Lehman, for which the name spiculisporic acid is now proposed, and (*b*) a hitherto undescribed acid for which the name minioluteic acid is proposed.

The evidence for the molecular constitution of minioluteic acid is briefly as follows:

(1) Minioluteic acid has the empirical formula $C_{16}H_{26}O_7$. It is a saturated dextrorotatory acid and titrates in the cold as a dibasic, and on heating as a tribasic acid. Because of the stability of the lactone ring the latter is probably of the γ-type.

(2) It gives a dimethyl ester and a trihydrazide.

(3) On fusion with potash, oxalic acid, *n*-dodecanoic acid, and decylsuccinic acid (II) $C_{14}H_{26}O_4$ but no succinic acid were isolated. Decylsuccinic acid has been synthesised.

(4) On reduction with sodium amalgam minioluteic acid gives the γ-lactone of α-hydroxy-γ-carboxy-*n*-tetradecanoic acid $C_{15}H_{26}O_4$ (III), the constitution of which has been settled by synthesis. This change involves the loss of a molecule of CO_2 and an atom of oxygen. The above facts are easily interpreted by assigning to minioluteic acid the molecular constitution (IV), *i.e.* the γ-lactone of αβ-dihydroxy-βγ-dicarboxy-*n*-tetradecanoic acid.

The origin of the products given on potash fusion and on reduction with sodium amalgam may be readily explained by postulating the formation of an intermediate product α-keto-βγ-dicarboxy-*n*-tetradecanoic acid (V) (or its corresponding enol form) from minioluteic acid in alkaline solution by loss of a molecule of water. The conversion in aqueous solution of αβ-dihydroxy-compounds, particularly of aliphatic nature, into the corresponding aldehyde or ketone by loss of a molecule of water is a well-known reaction [Lieben, 1902].

If the intermediate formation of α-keto-βγ-dicarboxy-*n*-tetradecanoic acid (V) be accepted the origin of the products isolated becomes obvious. Since the keto-group in this compound is in the β-position with regard to the β-carboxy-group this substance should behave as a β-keto-acid. By analogy with aceto-acetic acid, which on hydrolysis with strong potash gives rise to two molecules of acetic acid, α-keto-βγ-dicarboxy-*n*-tetradecanoic acid (V) under similar conditions (*i.e.* potash fusion) should yield oxalic and decylsuccinic acids. The latter may be presumed to break down further in presence of oxygen and afford *n*-dodecanoic and oxalic acids in the same way as succinic acid, which under these conditions gives oxalic acid. This accounts for all the degradation products isolated after subjecting minioluteic acid to potash fusion.

On hydrolysis with dilute alkali α-keto-βγ-dicarboxy-*n*-tetradecanoic acid (V) would be expected to give rise to α-keto-γ-carboxy-*n*-tetradecanoic acid and CO_2, by elimination of the β-carboxy-group, just as acetoacetic acid under these conditions affords acetone and CO_2. Reduction with sodium amalgam would then convert the keto-group into a hydroxy-group, the final product being α-hydroxy-γ-carboxy-*n*-tetradecanoic acid (III). This hypothesis is confirmed by the method used for synthesis of α-hydroxy-γ-carboxy-*n*-tetradecanoic acid since the decarboxylation and subsequent reduction of α-keto-βγ-dicarboxy-*n*-tetradecanoic acid, which must here be the first product of synthesis, follows the same course. The two typical methods of breakdown of a β-keto-acid are thus represented.

A comparison of the constitutional formulae assigned to spiculisporic and minioluteic acids reveals the fact that both are mono-γ-lactones of tricarboxylic acids. Considering for simplicity the hydrolysed, non-lactonic forms, it is seen that both contain the *n*-dodecanoic acid grouping, but whereas in spiculisporic acid this is linked by the α-carbon through a carbinol group with succinic acid, in minioluteic acid it is linked, again by the α-carbon, to dihydroxysuccinic (*i.e.* tartaric) acid, but without the intervention of a carbinol group.

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CXIX. NOTE ON THE LOSS OF NITROGEN AND SULPHUR ON DRYING FAECES.

By DAVID PATON CUTHBERTSON
and ALEXANDER KING TURNBULL.

From the Biochemical Laboratory of the Royal Infirmary and the Pathology Department, The University, Glasgow.

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When the collection of faeces during a metabolic experiment extends over a considerable period of time, difficulties arise in the determination of the total nitrogen and sulphur contents. Sampling of the wet composite specimen is generally unsatisfactory and ordinary methods of drying on a steam-bath result in a considerable loss of both nitrogen (as ammonia) and sulphur (mainly as sulphide).

If nitrogen is the only element under consideration its loss as ammonia can be prevented by acidification with H_2SO_4 before drying. To obviate drying Van Slyke [1932] has recommended sampling from a suspension of the faeces in H_2SO_4. If sulphur is also being determined, the excess of sulphuric acid which requires to be added masks the sulphur originally present in the faeces, and further, acidification leads to loss of H_2S and certain organic sulphides on drying.

The following experiments indicate the extent of the nitrogen and sulphur loss during drying. A method of preventing this loss is described.

Experimental.

Total N was estimated by the Kjeldahl method; total S by Denis's modification of Benedict's ashing method. Analyses were performed in triplicate, first on the wet specimens and then on aliquot portions subjected to various procedures. The accuracy of the sampling of these wet specimens was confirmed by the close agreement of triplicate estimations.

All specimens of human faeces were analysed within an hour or two of being voided.

Exp. 1. Sample of faeces containing 0·8976 g. total N had after 10 days' continuous drying on a steam-bath 0·8439 g. N. % loss = 5·9.

Exp. 2. Sample of faeces containing 0·616 g. N had after 14 days' drying on a steam-bath 0·558 g. N. % loss = 9·4.

Exp. 3. Sample of faeces containing 1·599 g. N and 0·222 g. S contained after 14 days' continuous drying on a steam-bath 1·531 g. N and 0·116 g. S. % loss N = 4·2. % loss S = 47.

Exp. 4. Fresh wet faeces containing 1·305 g. N were transferred to a vacuum-pan and the temperature of the bath raised to 80°. Ammonia-free air was passed over the faeces and drying was carried out under reduced pressure. In the course of 1 hour during which the temperature of the bath was rising no ammonia came over. In the next 5 hours 0·0286 g. N was trapped in *N*/10 H_2SO_4. That the loss of N was as NH_3 was confirmed by nesslerisation. During the subsequent 6 hours only 0·0007 g. more N came over. Loss of N = 2·2 % in course of 14 hours' drying.

In an attempt to prevent the simultaneous loss of N and S from faeces by means of a preliminary treatment with substances which would trap NH_3 and H_2S no simple method could be devised. It was eventually determined that the most satisfactory method was to moisten the faeces with excess 2 % copper acetate and trap the ammonia in acid during the process of drying.

Exp. 5. Sample of faeces containing 0·114 g. S contained after drying with copper acetate 0·115 g. S. There was thus no loss of S. When an aliquot portion was dried without any precaution for a similar period, *viz.* 2 days, the dried faeces contained 0·109 g.—a loss of 4·8 % S.

From clinical and bacteriological considerations it is obvious that the amounts of free gases contained in faeces must vary considerably.

Exp. 6. A sample of faeces containing 1·867 g. N was dried in the presence of 2 % copper acetate for 24 hours. Ammonia-free air was passed over the faeces and then through standard H_2SO_4. Ammonia was estimated by nesslerisation as it was found that acetic acid was also driven over. The total amount of trapped N was 0·275 g. The total residual N was 1·591 g. 1·866 g. N of the original 1·867 g. N were thus accounted for.

The original sample of faeces contained 0·1705 g. S. An aliquot dried without precaution for 24 hours contained 0·147 g. S and one dried for 6 days contained 0·1355 g. S—losses of 14 % and 20 % respectively. An aliquot treated with copper acetate lost no S on drying for 24 hours.

Exp. 7. A sample of faeces containing 2·522 g. N was dried in presence of 2 % copper acetate for 48 hours. Ammonia-free air was passed over the faeces and through a 10 % NaOH trap to absorb the acetic and other volatile acids. The air was finally passed through standard acid. In the final estimation the residual ammonia was distilled over from the NaOH solution and added to that trapped in the acid. The total loss was 0·0339 g. N. The nitrogen content of the dried faeces was 2·495 g. The original 2·522 g. were thus accounted for.

The original sample of faeces contained 0·588 g. S. An aliquot dried without precaution for 24 hours contained 0·581 g. S and one dried for 5 days 0·5675 g. S—losses of 1·2 % and 3·5 % respectively. An aliquot dried with 2 % copper acetate lost no S on drying for 24 hours.

In these experiments allowance was made for the trace of S in the copper acetate by adding aliquot amounts at the appropriate times. It was thus added to ordinary air-dried specimens of faeces after the process of drying was complete but before analysis.

It is seen from these experiments that an accurate analysis of the nitrogen and sulphur of faeces is possible if in the process of drying, ammonia-free air is passed over the faeces to which have been added a dilute solution of copper acetate in excess, the air is then passed through alkali to trap acetic and other volatile acids and then through standard acid. The alkaline fluid traps some ammonia and this is finally distilled into the excess standard acid.

SUMMARY.

1. The process of drying faeces on a water-bath leads to a considerable loss of N (mainly as ammonia) and S (mainly as sulphide).

2. This loss can be prevented by adding an excess of a dilute solution of copper acetate to the faeces before drying them in a closed vessel through which passes a current of dry ammonia-free air. The ammonia liberated from the faeces during drying is trapped in acid.

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REFERENCE.

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CXX. THE FATTY ACIDS OF PIG LIVER.

I. THE OCTADECENOIC ACIDS AND THE DESATURATION THEORY.

By HAROLD JOHN CHANNON, EILEEN IRVING and JAMES ANDREW BUCHAN SMITH.

From the Department of Biochemistry, The University of Liverpool.

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The accumulation of fat which occurs in the liver under the action of many substances, which is seen in numerous clinical conditions and which may be produced also by dietary methods, is the outstanding proof of the fact that the liver plays a fundamental part in fat metabolism. The desaturation theory, associated with the names of Leathes and Hartley, according to which fatty acids of the depot fat are transported to the liver, where they are desaturated and passed on to the tissues in this more readily oxidisable form, was put forward in an attempt to interpret this phenomenon. This theory is based on two lines of evidence: the effect of the food fat on the nature of the liver fatty acids [Leathes and Meyer-Wedell, 1909] and the investigations of Hartley [1909] on the unsaturated acids of the liver and depot fat of the pig. Considerable evidence has been put forward recently which suggests that the basis on which the desaturation theory rests is far less secure than had appeared. The demonstration by Leathes and Meyer-Wedell [1909] that the iodine value of the liver acids of cats and rats, which had been fed on highly unsaturated fish oils, might exceed that of the acids of the oil administered, was interpreted as evidence for desaturation. The extensive work of Sinclair [1932, 1, 2] seems to lend considerable support to the suggestion made by Joannovics and Pick [1910] that the increase in iodine value of the liver fatty acids caused by feeding oils of high iodine value observed by Leathes and Meyer-Wedell was due not to desaturation but to preferential absorption of the more unsaturated acids from the portal circulation. Sinclair has shown that the iodine value of the fatty acids of the liver phosphatides is rapidly raised by the administration of very small amounts of a highly unsaturated fat such as cod-liver oil, whilst the lowering of this high value by substitution of the cod-liver oil by a relatively saturated fat takes a much longer time. These and many other results of Sinclair imply an ability on the part of the tissues and particularly of the liver preferentially to take up the more unsaturated acids from the fat present in the food and tenaciously to retain them. At the present time therefore the selective retention by the liver cells of the more unsaturated acids seems probable and, if this can be established, one of the lines of evidence on which the desaturation theory rests will be invalidated. The second and perhaps more conclusive work which points to liver desaturation of fatty acids was provided by Hartley [1909] who isolated from the liver acids of the pig a $\Delta^{12,13}$ oleic acid and a $\Delta^{9,10;12,13}$ linoleic acid, while the ordinary $\Delta^{9,10}$ oleic acid was absent. Neither of these two former acids was present in lard which, however, was found to contain

$\Delta^{9,10}$ oleic acid and stearic acid. Hartley concluded that the liver cells possessed the power of introducing a double bond into the 12 : 13 position, so that on mobilisation to the liver stearic acid was converted into $\Delta^{12,13}$ oleic acid and similarly $\Delta^{9,10}$ oleic acid was converted into $\Delta^{9,10;12,13}$ linoleic acid.

This attractive evidence seems strong support for the liver desaturation theory, especially as the $\Delta^{12,13}$ oleic acid was unlikely to have arisen from the food fat, for such an acid has not been found in natural products. It was perhaps surprising, therefore, when Turner [1930] failed to obtain any evidence for the presence of this particular oleic acid in sheep liver, in which only $\Delta^{9,10}$ oleic acid was found to be present. In spite of the conflicting evidence which the work of Sinclair seems to provide and the doubt suggested by the results of Burr and Burr [1929; 1930; Burr *et al.*, 1932], which will be mentioned later, confirmation of the proof of the presence of this $\Delta^{12,13}$ oleic acid in liver would yet provide strong evidence for the desaturation hypothesis. We have undertaken an investigation of the fatty acids of pig liver with this object in view.

Experimental.

Preparation of the liver fatty acids.

The preparation of the crude fatty acids was carried out industrially in the following manner. 100 kg. of pig liver obtained direct from the slaughter-house were minced and mixed with a solution of potassium hydroxide. The mixture was allowed to stand until dissolution had occurred, when it was extracted six times with a non-chlorinated industrial solvent mixture. The material left on evaporation of the solvent (unsaponifiable matter and unhydrolysed lipoid substances) was saponified with alcoholic potash and the unsaponifiable fraction removed. The alkaline liver liquors (320 l.) were acidified with hydrochloric acid and the fatty acids removed by solvent extraction. The soap solutions resulting from the saponification and extraction of the unsaponifiable fraction were acidified and treated similarly. The crude fatty acids as received by us weighed 4·37 kg. and contained a considerable amount of the higher-boiling fractions of the solvent used for extraction. Contaminating impurities were removed by dissolution of the crude material in light petroleum (40 l.) and filtration after standing overnight. Evaporation of the light petroleum was followed by removal of the bulk of the industrial solvent by distillation on a boiling water-bath at 1 mm. The partially purified material was then saponified in the usual way with alcoholic potassium hydroxide. Removal of the unsaponifiable fraction by extraction with ether removed also the remainder of the industrial solvent. The fatty acids after purification by treatment with light petroleum weighed 1·835 kg. (I.v. 110, mol. wt. 229). The yield, 1·84 % of the fresh liver weight, is probably about 60 % of the total acids originally present in the liver in different forms. The extraction of acidified liver tissue after hydrolysis is an extremely difficult operation, especially in bulk, and the yield obtained therefore is to be regarded as satisfactory. Such a yield makes it unlikely that the material is not representative of the whole.

Removal of the saturated acids.

The oxidations described later were carried out after the removal of the saturated acids either from the bulk preparation described above or from various small laboratory preparations made by exhaustive alcohol extraction of minced liver with subsequent preparation of the fatty acids from the alcoholic extract. The Twitchell process, as modified by Hilditch and Priestman [1931], applied

to 5·033 g. of the bulk preparation (I.V. 110, mol. wt. 299) gave 1·675 g. of saturated acids (I.V. 2·5, yield 33·3 %). A similar result was obtained on the large scale. 670 g. of the same acids yielded 231 g. of saturated acids (I.V. 2, mol. wt. 277, yield 34 %) and 440 g. of unsaturated acids (I.V. 153, mol. wt. 321). The proportion of saturated acids in the other preparations was of the same order, the highest figure being 39 %.

The preparation of the dihydroxy-acids from the unsaturated fraction.

Since the main object of this work was to establish the structure of the acids containing one double bond, it was desirable to explore the available methods for oxidation in order to determine the conditions under which greatest yields of dihydroxy-acids could be obtained. By oxidation of pure oleic acid in 0·1 % solution with alkaline potassium permanganate at 0° for 5 minutes, Lapworth and Mottram [1925, 1] obtained 95 % of the theoretical yield of dihydroxystearic acid. These authors state that the yield is depressed if the oxidation is carried out in the presence of more than a certain maximum of less saturated acids. The liver unsaturated acids consist of a complex mixture of acids from oleic acid to acids of more than twenty carbon atoms and having four and five double bonds. When such a mixture is oxidised under the conditions in which the greatest yield is obtained from pure oleic acid, the yield of dihydroxy-derivatives may clearly be seriously diminished. In order to determine the optimum conditions a series of comparative experiments, in all of which oxidation by alkaline permanganate has been used, have been carried out on the unsaturated fraction itself, and also on the material remaining after various processes have been carried out in attempts to concentrate the acids containing one double bond. Before describing these experiments, mention is made of the fact that when a mixture of acids, which has been freed from unsaponifiable matter, is oxidised by alkaline permanganate, there are produced substances which may be extracted by ether from the alkaline solutions of the oxidation products. The nature of this unsaponifiable fraction generated during oxidation has not been investigated. It is a complex mixture derived from the more unsaturated acids. It has been removed at various stages of the oxidations and it is mentioned here to explain the appearance of unsaponifiable fractions in the results which follow.

The method of Lapworth and Mottram [*1925, 1*]. 19·07 g. of the unsaturated fraction (I.V. 193) from a small laboratory preparation of fatty acids were dissolved in 20 l. of ice-cold water containing 14·0 g. potassium hydroxide. 14·6 g. potassium permanganate dissolved in 700 ml. ice-cold water were now added quickly with stirring, and after 5 minutes the oxidation was stopped by the addition of concentrated hydrochloric acid and sodium bisulphite solution. After removal by filtration, the insoluble oxidation products were dried *in vacuo* at room temperature over phosphorus pentoxide and extracted with light petroleum (B.P. 80–100°) to remove unoxidised acids and unsaponifiable material. The undissolved residue consisting of di- and tetra-hydroxy derivatives was then separated into its two main components by boiling with ether, the tetra-derivative being practically insoluble in that solvent. The portion soluble in light petroleum (6·54 g.) had I.V. 59·3 and mol. wt. 444. From it 1·03 g. of unsaponifiable matter were removed, yielding a mixture of acids of mol. wt. 383 and I.V. 69. 4·38 g. of these acids soluble in light petroleum were now re-oxidised under exactly the same conditions. The oxidation products insoluble in water

were filtered off, dried and extracted with petroleum. The petroleum-soluble portion was oxidised a third time. The combined yields of the various products from the three oxidations were: dihydroxy-derivatives 2·06 g.; tetrahydroxy-derivatives 0·70 g.; a semi-solid mixture of saturated acids soluble in petroleum 2·393 g. Allowing for the removal of portions of the oxidation products for analytical purposes, the yield of dihydroxy-derivatives was 13·0 % of the unsaturated acids or 8·0 % of the original mixed acids from the liver.

The method of Hartley [1909] used by Turner [1930]. The essential difference in the method used by these workers, as compared with that of Lapworth and Mottram, was that they carried out their oxidations in solutions which were approximately 13 times more concentrated. Following their procedure, 9·28 g. of the unsaturated acids (I.V. 193) were dissolved in 92 ml. 4 % potassium hydroxide, 280 ml. water were added and, after cooling to 0°, the mixture was oxidised by the addition of 400 ml. of 1·5 % potassium permanganate solution also at 0°. The oxidation was terminated after 10 minutes by treatment as in the previous experiment. A second oxidation of the petroleum-soluble fraction of the oxidation products was carried out under similar conditions. The final yields were: dihydroxy-derivatives 0·20 g.; tetrahydroxy-derivatives 0·15 g. This amount of dihydroxystearic acid corresponds to 2·5 % and 1·5 % of the unsaturated and of the original mixed acids respectively. The corresponding figures for the Lapworth and Mottram process were 13·0 and 8·0 %. The latter process, carried out at great dilution, is clearly far more efficient for the quantitative isolation of hydroxy-derivatives of the singly unsaturated acids present in liver. Lapworth and Mottram's statement as to the effect of the presence of more highly unsaturated acids in depressing the yield of dihydroxy-acid from oleic acid in a mixture made it likely that this yield of 13 % might be considerably below the value obtainable if the more highly unsaturated acids were first removed. For this reason attempts were made to increase the yield by removing as much of the unsaturated acids as possible from the mixture. For this purpose, two methods were employed.

Removal of more unsaturated acids. When certain of the more unsaturated acids are brominated, isomeric soluble and insoluble bromides are produced. The latter may be removed and the soluble product debrominated. On rebromination a further amount of insoluble bromide is formed. This principle was employed in an attempt to free the acids containing one double bond from the more unsaturated acids, alternate bromination and debromination being employed until no further insoluble bromide could be obtained. 19·56 g. of the unsaturated acids (I.V. 193) brominated in ether at 0° yielded 7·48 g. of ether-insoluble bromo-acids and 35·58 g. of soluble products. The latter were dissolved in light petroleum (B.P. 40–60°) and further insoluble bromides, which were precipitated after standing for 24 hours at 0°, were removed. The petroleum-soluble fraction, 31·57 g., contained 47·1 % Br. 30·34 g. of this product on debromination by the method of Rollet [1909] yielded 14·05 g. of fatty acid of I.V. 132. On rebromination of 13·85 g. of these acids, a further 1·21 g. of ether-insoluble bromide was removed. The remainder (Br 43 %), which remained completely soluble in light petroleum after standing for 2 days at 0°, was debrominated as before. By repetition of this bromination-debromination process, there were finally obtained 10·62 g. of fatty acids of I.V. 98. 10·1 g. of the latter acids when oxidised by the method of Lapworth and Mottram yielded 1·98 g. of dihydroxy-products (M.P. 130° indefinite) and 0·429 g. of tetrahydroxy-products (M.P. 168° indefinite) the former yield corresponding to 10·9 % of the original unsaturated acids, as compared with a yield of 13 % by direct oxidation

of the unsaturated acids without preliminary application of the bromination process. Unsuccessful attempts were also made to apply the Twitchell process to the mixture of soluble brominated acids in an endeavour further to remove the polybromo-acids.

The second method used for removing the more unsaturated acids was the lithium soap process which has been frequently described in the literature in various forms (in particular Moore [1919]). 22·15 g. of unsaturated acids from the large preparation (I.V. 153) were dissolved in 90 ml. absolute alcohol and 1·8 g. lithium hydroxide dissolved in 22·5 ml. water were added. After standing at 0° overnight, about 3 g. of lithium soaps were removed. A further 1 g. of lithium hydroxide dissolved in 100 ml. 80 % alcohol was added and the mixture maintained at 0° for 24 hours. The combined alcohol-insoluble lithium soaps yielded 6·01 g. of acids (I.V. 96·1, mol. wt. 286, 27 % of the total) while 15·6 g. of the more unsaturated acids (I.V. 171, mol. wt. 327, 70·4 % of the total) were obtained by decomposition of the soluble soaps. 4·755 g. of the more saturated acids yielded on oxidation by the method of Lapworth and Mottram 2·68 g. of dihydroxy-compounds (2·40 g., M.P. 127°; 0·28 g., M.P. 112°) together with 0·21 g. tetrahydroxy-derivatives (M.P. 168°). 10·72 g. of the more unsaturated fraction yielded by similar oxidation 1·23 g. dihydroxy-compound (M.P. 126°) and 0·05 g. of tetrahydroxy-derivatives. For comparison with these results a sample of the original unsaturated acids (I.V. 153) was directly oxidised without being subjected to the lithium treatment. The results of this experiment are summarised and compared with others in Table II.

The effect of increasing the permanganate concentration on the yield of the oxidation products. In the oxidation experiments so far described, the proportions of the reagents used by Lapworth and Mottram were rigidly adhered to. Since the starting material used by these workers was oleic acid (I.V. 90·4), whereas the acids used in the present work were far more unsaturated, it was considered desirable to investigate the effect on the yield of the dihydroxy-fraction of increasing the permanganate concentration proportionally to the increase in the iodine value.

The results of all these variations of the conditions of oxidation on the yields of the products are set out in Tables I and II. Table I shows that the yield of dihydroxy-acids by the Lapworth and Mottram method is about five times as great as that obtained by Hartley's method, while the yield of tetrahydroxy-compounds is about two and a half times as great. The separation of the more unsaturated acids by bromination has given poorer yields of both products when compared with the direct oxidation by the former method. Increase in the permanganate concentration causes a fourfold increase in the yield of the tetrahydroxy-fraction (Exp. 6), but slightly depresses the yield of dihydroxy-products. Table II shows a slightly increased yield of the two products as a result of the lithium soap treatment (*cf.* 4 and 5). The increase in the amount of the dihydroxy-fraction, which was the main objective, was however insufficient to compensate for the extra labour involved in the lithium soap separation, and accordingly direct oxidation of the unsaturated acids was employed in the large scale preparation. Tables I and II show the variations which may occur in the proportions of singly unsaturated acids present in the mixed acids from different liver samples. In Exps. 2 and 5, in which the oxidations were carried out under identical conditions, but on different samples of acids, the yields of dihydroxy-products were 8·0 and 14·8 % respectively of the original mixed acids. Hartley [1909] obtained yields of dihydroxy-compounds varying from 2·6 to 4·5 % of the mixed pig liver acids.

Table I. *Yields of di- and tetra-hydroxy-acids obtained under various conditions of oxidation.*

The unsaturated acids used in these experiments formed 61·5 % of the total mixed acids, and had I.V. 193.

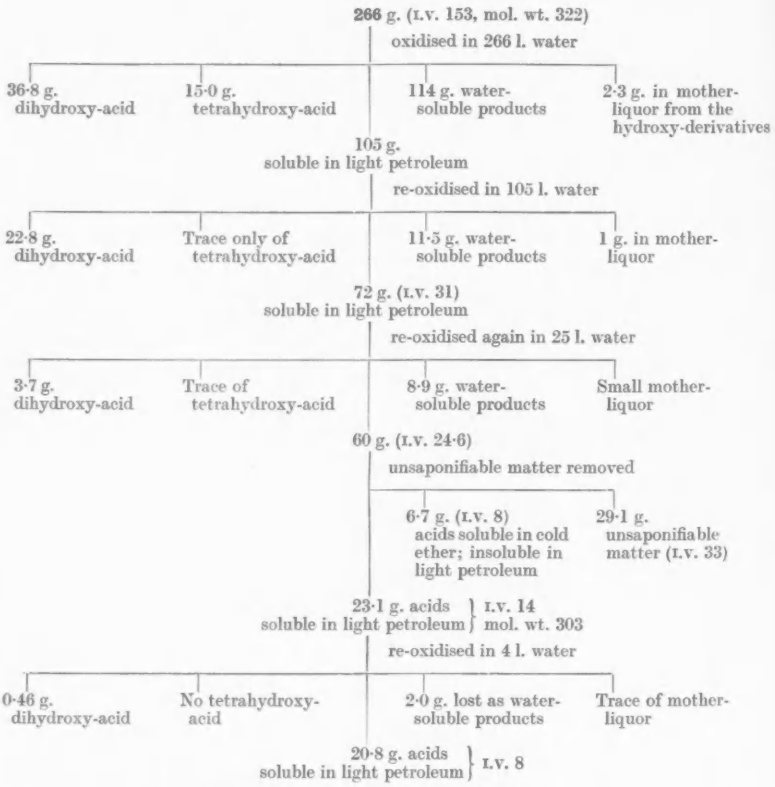
Method used	% yield from unsaturated acids		% yield from original acids (calc.)	
	Di-hydroxy	Tetra-hydroxy	Di-hydroxy	Tetra-hydroxy
1. Hartley's method of oxidation of unsaturated acids	2·5	1·6	1·5	1·0
2. Lapworth and Mottram's method on the unsaturated acids	13·0	4·2	8·0	2·6
3. The singly unsaturated acid in the mixed unsaturated acids first concentrated by bromination experiments and then oxidised by Lapworth and Mottram's process	11·0	2·4	7·0	1·5

Table II. *Yields of di- and tetra-hydroxy-acids obtained by the Lapworth and Mottram method.*

The unsaturated acids used in these experiments constituted 66·1 % of the original liver acids and had I.V. 153.

Particular acids oxidised	% yield from acids from lithium soaps		% yield from total unsaturated acids		% yield from original mixed acids (calc.)	
	Di-hydroxy	Tetra-hydroxy	Di-hydroxy	Tetra-hydroxy	Di-hydroxy	Tetra-hydroxy
4. Acids from insoluble lithium soaps	56·3	4·4	15·65	1·2	10·34	0·8
Acids from soluble lithium soaps	11·5	0·5	8·3	0·33	5·5	0·2
Total by this method	—	—	23·95	1·53	15·84	1·0
5. Direct oxidation of mixed unsaturated acids	—	—	22·4	1·0	14·8	0·7
6. The same as the preceding, except that the permanganate has been increased	—	—	21·0	4·1	13·9	2·7

The preparation of the dihydroxy-acids on a large scale. 266 g. unsaturated acids (I.V. 153, mol. wt. 322) were oxidised in 266 l. water at 4°, with the proportions of potassium hydroxide and permanganate recommended by Lapworth and Mottram. The second oxidations of the material soluble in light petroleum were carried out in exactly similar manner. In the third and fourth oxidations the volume of water was decreased because of the low iodine value of the petroleum-soluble fractions which indicated that not more than a part of them could be oleic acid. The successive stages in the procedure are best illustrated by the scheme (p. 846), which is followed by a brief summary of the final results.


266 g. (I.V. 153, mol. wt. 322)
oxidised in 266 l. water
36·8 g. dihydroxy-acid
15·0 g. tetrahydroxy-acid
114 g. water-soluble products
2·3 g. in mother-liquor from the hydroxy-derivatives
105 g. soluble in light petroleum
re-oxidised in 105 l. water
22·8 g. dihydroxy-acid
Trace only of tetrahydroxy-acid
11·5 g. water-soluble products
1 g. in mother-liquor
72 g. (I.V. 31) soluble in light petroleum
re-oxidised again in 25 l. water
3·7 g. dihydroxy-acid
Trace of tetrahydroxy-acid
8·9 g. water-soluble products
Small mother-liquor
60 g. (I.V. 24·6)
unsaponifiable matter removed
6·7 g. (I.V. 8) acids soluble in cold ether; insoluble in light petroleum
29·1 g. unsaponifiable matter (I.V. 33)
23·1 g. acids soluble in light petroleum } I.V. 14 mol. wt. 303
re-oxidised in 4 l. water
0·46 g. dihydroxy-acid
No tetrahydroxy-acid
2·0 g. lost as water-soluble products
Trace of mother-liquor
20·8 g. acids soluble in light petroleum } I.V. 8

N.B. Water-soluble products are calculated by difference.

The results are summarised in Table III.

Table III.

Wt. of unsaturated acids used	266 g.,	equivalent to	402 g. of original mixed acids
,, crude dihydroxy-derivatives	63·8 g.,	,,	56·9 g. of "oleic" acid
,, crude tetrahydroxy-derivatives	15·0 g.,	,,	12·1 g. of "linoleic" acid
,, saturated acids	20·8 g.		
,, acids soluble in cold ether, insoluble in light petroleum*	6·7 g.		
,, unsaponifiable matter*	29·1 g.		
,, water-soluble products *etc.* (by difference)†	140·4 g.		

* The nature of these oxidation products was not investigated further.

† These products would consist mainly of a mixture of the further cleavage products of small molecular weight derived from all the different unsaturated acids, together with the hydroxy-derivatives of the highly unsaturated acids (C_{20} and C_{22}).

Investigation of the dihydroxy-fraction.

In view of the possibility that the three yields of dihydroxy-acids obtained in the successive oxidations, 36·8, 22·8 and 3·7 g. respectively, might differ in nature on account of preferential oxidation of one or more acids, each was

separately fractionally crystallised from a mixture of alcohol:ether (1:1). The yields and properties of the resultant six fractions are recorded in Table IV.

Table IV. *Fractionation of the dihydroxy-acids.*

	Fraction	Weight g.	M.P. ° C.	C %	H %	Mol. wt. (titration)
From first oxidation	1*a*	28·7	126 –126·5	67·75	11·3	314
36·8 g.	1*b*	5·2	119·5–122 (indef.)	67·1	11·2	314
From first re-oxidation	2*a*	15·3	129·5–130·5	68·4	11·45	312
22·8 g.	2*b*	5·6	117·5–120·5	68·5	11·4	315
From second re-oxidation	3*a*	2·1	128·5–129	68·5	11·5	317
3·7 g.	3*b*	1·2	117·5–120·5	68·6	11·4	315
$C_{18}H_{36}O_4$ requires				68·35	11·5	316

The analytical figures for fractions 1*a* and 1*b* suggest the presence of some tetrahydroxy-acid caused by the slight solubility in the very large volumes of boiling ether which were used in the separation of the crude di- and tetrahydroxy-acids from each other. These fractions were accordingly combined, extracted with the minimum volume of boiling ether and the soluble dihydroxy-product was then fractionally crystallised from alcohol-ether. Following the removal in this way of 5 g. of crude tetrahydroxy-acid from fraction 1, all the fractions were successively crystallised from this solvent, and by this means the material was finally divided into a series of fractions with the following compositions and properties:

Table V. *Composition and properties of the dihydroxy-acids.*

Fraction No.	Weight g.	M.P. ° C.	C %	H %
1	36·0	130·5–131	68·4	11·3
2	1·3	128	68·1	11·3
3	0·8	124–125	68·4	11·6
4	8·1	117–120	68·45	11·4
5	1·7	114	67·7	11·2
$C_{18}H_{36}O_4$ requires			68·35	11·5

Table V shows that at least 70 % of the dihydroxy-acids consist of a compound melting at 130·5–131°. The melting-points and combustion analyses of fractions 2–5 imply the existence of three compounds: one, M.P. 130·5–131° (fraction 1); another of low carbon content, M.P. 114° (fraction 5); and an intermediate compound which constitutes the main bulk of the material, apart from fraction 1, namely fraction 4, 8·1 g., M.P. 117–120°. Fraction 4 was repeatedly recrystallised from various solvents, but no effective further purification was obtained. This fraction appears to be a mixture of two dihydroxystearic acids, while fraction 5 is possibly the dihydroxy-derivative of an acid of lower carbon content.

The constitution of dihydroxystearic acid, M.P. 130·5–131°.

This was investigated by the method of Lapworth and Mottram. To 10 g. of the acid dissolved in 6 l. water containing 30 g. potassium hydroxide cooled to below 6° were added 4 l. 1 % aqueous potassium permanganate also cooled to below 6°. The mixture was well shaken and after remaining at room temperature for 4 hours the oxidation was stopped by the addition of sodium bisulphite solution and concentrated hydrochloric acid. A slight precipitate,

formed at this stage was filtered off. It weighed 0·12 g. and melted at 126° and was therefore probably unchanged dihydroxystearic acid. The amount of acid actually oxidised was therefore 9·88 g. The aqueous filtrate after neutralisation with sodium carbonate was reduced to a volume of 900 ml. and was then extracted thoroughly with ether.

Monobasic acid. The residue left after the removal of the ether was distilled in steam for 3 hours. The distillate was extracted with light petroleum (B.P. 40–60°). The solution, first dried over sodium sulphate, yielded on evaporation of the solvent 3·965 g. of liquid acids. 3·81 g. of these acids were distilled. 3·332 g. distilled at 234°. Further fractionation yielded 2·334 g. having B.P. 238° and mol. wt. (determined by the silver salt method) 144·2, 142·6. The zinc salt was prepared (found: C, 54·7; H, 8·5 %). The small amount of acid boiling above 240° was not further investigated. The theoretical figures for *n*-octanoic and *n*-nonanoic acids are as follows:

	Yield g.	B.P. °C.	Mol. wt.	Of zinc salt C %	Of zinc salt H %
n-Octanoic acid	4·50	236–237	144	54·6	8·6
n-Nonanoic acid	4·91	253–254	158	56·9	9·0

Dibasic acid. The residue from the distillation in steam (about 70 ml.) was filtered while hot from traces of tar and the dibasic acid allowed to crystallise; yield 4·29 g., M.P. 135–136° with previous softening. Suberic acid requires 5·44 g., M.P. 141°. On concentrating the aqueous filtrate, a further 0·28 g., M.P. 120° (indefinite) was obtained. These products were divided into four fractions by crystallisation from ether.

1. 2·522 g. M.P. 140·5–141°; mol. wt. 174·0; C, 55·3; H, 8·1 %.
2. 1·083 g. M.P. 140–141°.
3. 0·218 g. M.P. 135–137°.
4. 0·27 g. M.P. 100° indefinite.

Suberic acid, $C_8H_{14}O_4$, requires C, 55·15; H, 8·1 %; mol. wt. 174; M.P. 141°. The fourth fraction was converted to its magnesium salt, which was crystallised from water. The least soluble portion yielded 0·05 g. of acid of M.P. 102·5°. Azelaic acid melts at 106°. The oxidation products were therefore suberic and *n*-octanoic acids, together with small amounts of the acids having one more carbon atom, *i.e.* azelaic and *n*-nonanoic acids. In their oxidation of pure oleic acid, Lapworth and Mottram [1925, 2] obtained an 80 % yield of suberic acid and a 70 % yield of *n*-octanoic acid. They did not isolate azelaic or *n*-nonanoic acids from their oxidation products. They also estimated the amount of oxalic acid produced from the carbon atoms adjacent to the double bond. This was not sought in our experiments. These results show therefore that at least 70 % of the singly unsaturated acid present in this sample of pig liver is $\Delta^{9,10}$ octadecenoic acid.

Investigation of the dihydroxystearic acid, M.P. 117–120°.

4·048 g. of this product were oxidised and the products isolated in exactly the same way as in the previous experiment. None of the substance remained unoxidised.

The monobasic acid. This weighed 1·505 g.; mol. wt. 145. 1·252 g. on distillation gave three fractions, (*a*) 0·5 g., B.P. up to 220°; (*b*) 0·41 g., B.P. 221–235°; (*c*) 0·27 g., B.P. 236–240°. By redistillation of fractions (*a*) and (*b*) there were

obtained (i) 0·31 g., B.P. 220°; (ii) 0·28 g., B.P. 221–235°. The mol. wt. of fraction (i), determined by the silver salt method, was 136·4. This together with its boiling-point precludes the presence of *n*-hexanoic acid. The zinc salt was prepared from fraction (*b*) and gave on analysis C, 55·5; H, 8·8 %. The theoretical yields and the mol. wts. and boiling-points of the possible acids which might be present are:

Acid	Yield	Mol. wt.	B.P. ° C.
n-Nonanoic	2·02	158	253–254
n-Octanoic	1·84	144	236–237
n-Heptanoic	1·66	130	224
n-Hexanoic	1·49	116	206
Crude product (found)	1·505	145	up to 240

With the small amount of material available, it was not possible to carry the investigation further. Study of the boiling-points and molecular weights of the various fractions, together with the initial weight of the crude oxidation product, make it appear certain that *n*-hexanoic acid could not have been present unless in traces. The mixture of acids probably consisted of *n*-heptanoic, *n*-octanoic and *n*-nonanoic acids.

The dibasic acid. The aqueous residue from the distillation in steam was filtered from 0·24 g. of a dark brown oil and from the filtrate there crystallised 1·73 g. dibasic acid, M.P. 126–129°, clearing finally at 132°. On concentrating the mother-liquor, a second crop of 0·28 g. separated, M.P. 120° (indefinite). Total crude dibasic acid, 2·0 g. The theoretical yields of sebacic, azelaic and suberic acids would have been 2·59 g., 2·41 g. and 2·23 g. respectively. It is to be noted that in both crops the product differed in melting-point from that obtained at the corresponding stage from the oxidation of the higher-melting dihydroxystearic acid (p. 848). On recrystallisation from ether, the first crop still melted at 120–125° and had mol. wt. 175. Further recrystallisation from ether had no apparent effect. Both fractions were therefore converted into their magnesium salts and these fractionally crystallised from water. The acids from the most and from the least soluble salts were obtained and recrystallised both from water and from ether. The most soluble magnesium salt gave a dibasic acid, which, after recrystallisation, weighed 0·32 g.; M.P. 139–141°; C, 55·15; H, 8·1 %; mol. wt. 175 (by titration). The acid from the least soluble magnesium salt weighed 0·28 g., crystalline plates, M.P. 133–134°; C, 59·60; H, 8·9 %; mol. wt. 206. The theoretical data for four possible dibasic acids are:

	M.P. ° C.	C %	H %	Mol. wt.
$C_{11}H_{20}O_4$	124	61·1	9·3	216
$C_{10}H_{18}O_4$	134	59·4	8·9	202
$C_9H_{16}O_4$	106	57·4	8·5	188
$C_8H_{14}O_4$	141	55·15	8·1	174

The dibasic products of oxidation therefore contain suberic and sebacic acids; and it follows that in all probability azelaic acid is a third component, because, apart from any other consideration, suberic and sebacic acids present alone in a mixture would be very readily separated, whereas these two components were only obtained in the present instance with difficulty. The presence of other dibasic acids in very small traces is also possible. In Table VI are given the products which would arise from a number of acids oxidised under the conditions used by us. The presence of suberic and *n*-octanoic acids and also of part of the azelaic and *n*-nonanoic acids in the oxidation products in this experiment shows

Table VI.

Acid	Main products	By-products
9:10-Dihydroxystearic acid	Suberic and *n*-octanoic acids	Azelaic and *n*-nonanoic acids
10:11 „	Azelaic and *n*-heptanoic acids	Sebacic and *n*-octanoic acids
11:12 „	Sebacic and *n*-hexanoic acids	Nonamethylenedicarboxylic acid and *n*-heptanoic acid
12:13 „	Nonamethylenedicarboxylic acid and *n*-pentanoic acid	Decamethylenedicarboxylic acid and *n*-hexanoic acid

that the lower-melting dihydroxy fraction is a mixture and that the $\Delta^{9,10}$ dihydroxy-derivative has not been entirely removed by fractional crystallisation. The yields and analytical data suggest that about one-half of the fraction may be the 9 : 10 compound. It therefore remains to account for the apparent formation of sebacic and *n*-heptanoic acids. Under the conditions of oxidation used, the presence of these products suggests that the hydroxy-groups were originally in the 10 : 11 positions. This view, however, can only be put forward with considerable caution. Until the oxidation products can be obtained in far greater quantity to enable their isolation in pure form, no definite statement can be made. These results do however indicate the presence of an acid which is neither $\Delta^{9,10}$ nor $\Delta^{12,13}$ octadecenoic acid, and which probably forms not more than 10 % of the total dihydroxy-fraction.

Oxidation of ordinary oleic acid. At this stage it was felt wise to prepare the dihydroxystearic acid from ordinary oleic acid, in order to see whether it could be fractionated into high- and low-melting products of a similar nature. Accordingly 14·27 g. oleic acid (which still contained a little saturated acid) were oxidised by Lapworth and Mottram's method and yielded 13·27 g. dihydroxystearic acid, M.P. 128°. Allowing for the 1·67 g. of saturated acids which were removed by light petroleum, the yield was 94·0 % of theory. On fractionating the hydroxy-derivative as in the large experiment on p. 847 there were obtained 10·35 g., M.P. 130°; 1·0 g., M.P. 128° and 0·47 g., M.P. 122°, the mother-liquor of 1·4 g. being a semi-liquid product. This fractionation was very similar in its results to that described for the hydroxy-compounds from liver, except that in this case there was no corresponding lower fraction, with a melting-point of 117–120°, which remained unchanged after several recrystallisations.

Discussion.

These results show that at least 80 % of the octadecenoic acid of pig liver is ordinary oleic acid ($\Delta^{9,10}$), while some 10 % may be the $\Delta^{10,11}$ acid. No evidence was obtained of the presence of the $\Delta^{12,13}$ acid obtained by Hartley [1909]. This latter result is the more striking because the $\Delta^{12,13}$ acid was the only acid found in pig liver by that author. It was isolated by him as the dihydroxy-acid, M.P. 129°, which was further oxidised. From the steam-volatile oxidation products, calcium and barium salts were prepared and these contained the percentages of metal required by theory for *n*-hexanoic acid. It is to be noted however that the decamethylenedicarboxylic acid, which should have been produced under the conditions of oxidation used by him along with *n*-hexanoic acid, was not isolated. Hartley contrasts the melting-point of his dihydroxy-acid, M.P. 129·5°, with that obtained by Edmed [1898] by oxidation of oleic acid, which gave a dihydroxy-acid, M.P. 134°, as evidence for the dissimilarity of his compound from the $\Delta^{9,10}$ acid. Robinson and Robinson [1925], however, who record M.P. 132° for 9:10-dihydroxystearic acid prepared from

pure oleic acid, comment that the melting-points (up to 136°) previously recorded in the literature are too high.

Little can be said at present concerning the small fraction of the acid which was isolated in this work as a dihydroxy-acid, M.P. 117–120°. The material available in the work carried out by Hartley [1909] and Turner [1930] did not allow them fractionally to crystallise their dihydroxy-products, and no corresponding compound was obtained.

We feel that our results further weaken the available evidence for desaturation of fatty acids by the liver, for it will be recalled that Turner [1930] failed also to obtain evidence of the presence of any octadecenoic acid in sheep liver other than the $\Delta^{9,10}$ acid. Bearing in mind the extensive researches of Sinclair [1932, 1, 2] which show the remarkable avidity with which the liver removes unsaturated acids from the portal circulation and the tenacity with which it retains them when the unsaturated fat in the diet is substituted by a saturated one, the original results of Leathes and Meyer-Wedell can be interpreted equally well for the present as being due to preferential absorption of fatty acids or as being due to desaturation.

From another direction also considerable evidence is accumulating which seems to cast doubt on the validity of the desaturation hypothesis. The extensive literature which is accumulating to show that animals will not grow on a diet which is rigorously freed from fat, but that normal growth is restored when very small amounts of linoleic or linolenic acids are administered, has given rise to the conception of "essential" fatty acids which implies a very limited ability of the animal to synthesise certain unsaturated acids at least [Burr and Burr, 1929; 1930; Burr *et al.*, 1932]. If this finding is substantiated, it is of particular importance to emphasise that one of the "curative" acids is linoleic acid, which on the desaturation hypothesis should be produced from depot oleic acid by introduction of a double bond into the 12:13 position.

Summary.

1. With a view to obtaining further evidence on the theory of desaturation of fatty acids in liver, the octadecenoic acids of pig liver have been investigated by oxidation of the unsaturated acids and subsequent further oxidative degradation of the dihydroxy-acids.

2. Description is given of experiments carried out with a view to increasing the yields of dihydroxy-acids when the mixed unsaturated acids are oxidised with alkaline permanganate.

3. Fractional crystallisation of the latter acids shows the presence of at least two octadecenoic acids. One of these is shown to be $\Delta^{9,10}$ octadecenoic acid (ordinary oleic acid) and to constitute at least 85 % of the octadecenoic acids present. The second dihydroxy-acid, M.P. 117–120°, yielded on further oxidation a mixture of monobasic and dibasic acids which could not be satisfactorily separated on account of the small amount of material available. The evidence so far obtained from study of the oxidation products suggested the presence of a $\Delta^{10,11}$ acid.

4. The $\Delta^{12,13}$ octadecenoic acid isolated by Hartley [1909] from pig liver was shown to be absent.

5. The bearing of these results on the liver desaturation theory is discussed.

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CXXI. THE PHOSPHATIDES OF THE WHEAT GERM.

By HAROLD JOHN CHANNON
and CECIL ALFRED MAUNDER FOSTER.

From the Department of Biochemistry, The University of Liverpool.

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Investigations of the phosphatides of cabbage leaves have shown that the main constituent is phosphatidic acid, present as its calcium salt, and that lecithin and kephalin are absent [Chibnall and Channon, 1927]. This finding suggested that salts of phosphatidic acid might be the intermediate compounds in the synthesis of lecithin and kephalin from triglycerides, according to the scheme postulated by Trier [1912], and suggested further studies designed to throw light on this question. The work described in this paper was the first part of an investigation planned in order to study the phosphatides of a seed and to trace the changes which occur on germination. While it was in progress investigations along similar lines were reported by Smith and Chibnall [1932] and Jordan and Chibnall [1933]. The former workers identified lecithin, kephalin and the calcium or magnesium salt of phosphatidic acid as constituents of the phosphatide mixture obtained from grass; while the researches of the latter, designed along lines similar to our own, were concerned with an exhaustive study of the distribution of phosphatidic acid, lecithin and kephalin in the runner bean during the different stages of germination and growth. They established for the first time the presence of phosphatidic acid in a seed. Because of the difficulty of obtaining adequate amounts of material for fractionation, we chose wheat germ as our starting material, for it can be obtained commercially and by far the greater part of the phosphatide of the wheat grain is present in the embryo.

Experimental.

Extraction of the wheat germ.

As a result of studies of the phosphatide content of seeds, Schulze and Steiger [1889] recommended extraction by alcohol at 60°, the extract being evaporated to dryness and the residue extracted with ether, because they found that extraction of seeds directly with ether did not remove all the phosphatide. The advantage of the increased phosphatide yield obtained by this method tends to be offset by the fact that the final ethereal extract is contaminated by non-phosphatide substances, some of which may contain phosphorus. Jordan and Chibnall [1933] submitted the cotyledon residues of runner beans which had been already extracted with ether to this process. They found that much of the additional ethereal extract so obtained consisted of substances which readily lost their phosphorus on shaking an ethereal solution with dilute acid, and they consider that the extra amount of true phosphatide extracted by alcohol is not more than one-quarter of the total. With these findings in mind, we carried out an extensive series of extractions with ether, light petroleum, alcohol and acetone, alone and in mixtures, in order to obtain an extract of high phosphatide content and uncontaminated as far as possible. The degree

to which the phosphorus obtained in the extracts existed as non-phosphatide phosphorus was systematically investigated by the methods which were used for the large preparation and are described later, and only the method finally adopted as a result of these experiments need be described. Alcohol-ether (1:1) was found to be the most useful solvent for extraction, and a series of comparative experiments on one sample of wheat germ in which different volumes were employed was carried out. The germ was mixed with the solvent and the mixture heated to boiling. After standing for several hours, at room temperature, the mixture was filtered and the residue washed with successive volumes of ether until the filtrate was colourless (usually 4 volumes). The filtrate was evaporated to dryness *in vacuo* on a water-bath at 60° and the residue dissolved in anhydrous ether. After several hours' standing, the white sediment was removed by filtration and the ether-soluble material obtained by evaporation. The figures recorded below are typical of the results obtained:

Solvent	Volumes	Yield % of germ	P as % of germ
Ether (Soxhlet)	—	7·40	0·019
Alcohol-ether (1:1)	6	7·60	0·028
	9	7·80	0·025
	12	8·25	0·035
	20	8·53	0·036
	(mean from 6 different samples)		

Since our object was to obtain the phosphatide fraction in as great a yield as possible, extraction with 12 volumes of alcohol-ether as described above was finally adopted throughout this work. The yield of oil by this method was 8·40 % (mean of seven samples). Its phosphorus content corresponded to its containing about 10 % of phosphatide.

Precipitation of the phosphatide.

The removal of the phosphatide quantitatively from an oil in which it is present in relatively low concentration is difficult without the addition of inorganic salts. Because of a desire to avoid use of the latter, investigations were made of the optimum conditions for precipitation from ethereal solution by acetone. Samples from one preparation of wheat germ oil were dissolved in varying volumes of ether and to these solutions were added 9 volumes of acetone calculated on the volume of oil used. After cooling in the ice-box for several hours, the precipitates were filtered off and washed with acetone. From a phosphorus determination on the precipitate, the recoveries under the varying conditions were calculated. These are recorded in Table I. As a result of this experiment we adopted the method of dissolving the oil in 1·5 volumes of ether followed by precipitation with 9 volumes of acetone.

Table I. *Percentage of recovery of phosphatide on precipitation of ethereal solutions by acetone.*

	Ether-soluble P as % of the wheat germ	Phosphatide (P × 25) as % of oil or precipitate	Recovery of ether-soluble P in precipitate %
Original extract	0·0232	7·8	—
Precipitation by acetone (9 vols.) after dissolution in ether:			
Vols. 1	0·0202	54	87
1·5	0·0208	60	90
2	0·0191	60	82
3	0·0171	65	73

Analysis of a series of samples.

Before proceeding with the bulk preparation, a series of extracts from different samples of germ (400–850 g. each) was prepared by these methods in order to study the variations likely to be encountered. Their analyses are recorded in Table II.

Table II. *Analysis of seven samples of wheat germ oil.*

Sample	A	B	C	D	E	F	G	Mean
Age (days)	100 (kept at 4°)	42 (kept at 0°)	1	1	2	1	1	—
Yield g./100 g. germ	9·80	8·90	7·7	7·4	8·5	9·1	7·8	8·37
Acid value	—	—	—	17·5	21·7	14·1	16·1	17·6
Saponification value	181	188	190	192	184	180	—	184
I.V.	110	111	115	113	111	107	—	111
P % of extract	0·49	0·55	0·34	0·29	0·32	0·43	0·35	0·39
Fatty acids % of extract	83·2	80·0	82·3	84·7	83·4	82·6	83·8	82·8
I.V.	121	122	112	119	122	124	128	120
Mol. wt.	284	281	282	284	278	278	284	284
Unsaponifiable matter:								
Yield % of extract	6·8	8·0	9·2	5·6	6·7	6·1	6·8	7·03
Sterol (by digitonin)	55	50	47	68	60	65	—	57·5
I.V.	99	109	109	103	108	106	—	105·8

From Table II calculations may be made as to the approximate composition of the extract. Later in this paper it is shown that the phosphatide is present as calcium, magnesium and potassium phosphatidates, together with lecithin and kephalin, and that little of the phosphorus of the extract is non-phosphatide-phosphorus. If the mixed phosphatides be regarded as having a mean phosphorus content of 4 %, the approximate composition of the extract calculated on the mean values in Table II is:

Phosphatide $\left(\frac{0{\cdot}39}{4{\cdot}0} \times 100\right)$	9·75 %
Free fatty acid $\left(\frac{17{\cdot}6}{5{\cdot}6} \times 28{\cdot}2 \times 100\right)$	8·8 %
Glyceride (total fatty acids – phosphatide acids $(18{\cdot}2 \times \text{P})$ – free fatty acid) × 1·045	69·9 %
Unsaponifiable matter	7·03 %
Total	95·48 %

Thus about 4·5 % of the extract appears to be contaminating material of non-fatty nature. Analysis of the phosphatide fraction gave the results recorded in Table III.

Table III. *Analysis of the acetone-insoluble fractions from seven samples of wheat germ oil.*

	A	B	C	D	E	F	G	Mean
Yield as % of extract	13·7	15·9	8·0	10·2	10·4	18·7	12·6	12·8
Yield as % of germ	1·3	1·4	0·6	0·8	0·9	1·7	1·0	1·1
I.V.	77	78	74	78	78	75	—	77
Fatty acids %	55·3	52·6	57·2	57·7	53·3	56·4	—	56·2
I.V.	122	121	123	118	125	120	132	123·0
N %	1·8	1·8	1·5	1·5	1·5	1·6	1·5	1·60
P %	2·9	2·9	2·8	2·4	2·6	2·6	2·3	2·65
Ash %	2·4	2·6	—	2·7	3·2	2·6	—	2·70

The figures in Table III show that the mean value of the N:P ratio is 1·34, indicating the presence of contaminating nitrogenous substances which are so often found in the phosphatide-containing ethereal solutions of the residue left after evaporation of an alcoholic extract of tissues. Calculation on the basis of 4 % phosphorus shows that the mean value for the maximum amount of phosphatide possibly present is 66·25 %. This would yield on hydrolysis 48·32 % of fatty acid, calculated as oleic acid (P × 18·2), compared with 56·2 % found. The calculations from Table II, which indicate the presence of 4·5 % of non-lipoid substances in the original extracts, thus seem borne out by the presence of about 30 % of non-phosphatide in the acetone-insoluble fractions. Since these latter constituted 12·7 % of the original extracts, the 4·5 % would become 36 % if all the extraneous material of the extract were precipitated with the phosphatides. The phosphorus recovery in the acetone precipitates corresponds to 86·3 % of that originally present in the extracts. It is to be observed that the I.V. of the acids obtained by hydrolysis is identical with that of those yielded by the original material, which suggests that the composition of the phosphatide acids does not differ markedly from that of the glyceride acids.

Large scale preparation. 9·5 kg. of wheat germ, received fresh and kept in cold storage for 5 days, were extracted with 114 l. of ether-alcohol (1:1) in six batches. The preparation, carried out as described in 8 days, yielded 770 g. (8·5 %) of a brown oil which gave the following analytical values: acid value, 18·0; fatty acids (I.V. 125, mol. wt. 282), 85·3 %; unsaponifiable matter, 5·4 %; P, 0·39 %; all of which figures correspond closely with those recorded as the mean value in Table III.

Precipitation of the phosphatide.

Acetone (6·5 l.) was added to a solution of 747 g. in ether (1·2 l.). After standing for 15 hours in the refrigerator, the crude phosphatide was filtered off and washed with acetone. It was redissolved in ether (135 ml.) and reprecipitated by the addition of acetone (420 ml.). The product after washing with acetone weighed 68·9 g. (9·2 % of the extract). It analysed as follows: P, 2·97 %; N, 1·52 %; amino-N, obtained by Van Slyke apparatus after hydrolysis for 9 hours with *N* sulphuric acid, removal of the fatty acids and neutralisation after concentration, 0·15 %; the ash, 4·27 %, contained 29 % P and 12 % Mg. In order to ensure the absence of unsaponifiable matter which might have been carried down during the phosphatide precipitation, 0·517 g. was hydrolysed with sodium ethoxide for 4 hours. The yield of unsaponifiable matter was 0·032 g. or 6·20 %. This corresponds to 10·5 % of the unsaponifiable fraction present in the original extract. The fatty acids obtained after removal of the unsaponifiable fraction weighed 0·290 g. (56·1 %). These acids had I.V. 120 and mol. wt. 299.

From these data, and assuming that the ash represents pyrophosphate derived from metallic salts of phosphatidic acid present in the original mixture, an approximate distribution of the phosphorus may be derived as follows:

P present originally as salts of phosphatidic acid $\left(\text{ash } 4{\cdot}27\ \% \times \frac{29}{100}\right)$ ...	1·24 %
P present as kephalin $\left(\text{amino-N } 0{\cdot}15\ \% \times \frac{31}{14}\right)$	0·33 %
Residual P (2·97 − 1·24 − 0·33)	1·40 %
The residual P if present as lecithin would require $1{\cdot}4 \times \frac{14}{31}$ % N	0·63 %
The non-phosphatide-N, 1·52 − 0·78	0·74 %

On the basis of these figures, the composition of the mixture would be: lecithin, 36·4 %; kephalin, 8 %; phosphatidate calculated as the magnesium salt, 28·9 %; unsaponifiable matter, 6·2 %; giving a total of 79·5 %. Hence the fraction contains 20·5 % of extraneous substances which have a mean content of 3·6 % N. A phosphatide mixture such as the above would yield on hydrolysis 54·24 % fatty acids as against 56·1 % found. The phosphorus present in the phosphatide precipitate corresponds to not more than 70 % of that present in the original extract as compared with the mean figure of 86·3 % for samples A–G. We have no explanation to advance for this considerably lower figure.

Evidence was next sought as to the validity of the assumption concerning the nature of the ash and thereby whether the excess nitrogen as shown by the N:P ratio of 1·14:1 was due to extraneous nitrogenous substances present in the fraction or to the presence of a diaminophosphatide. The process finally adopted was that of emulsification of the phosphatide in water followed by aggregation by the addition of acetone and salt, a modification of that used by MacLean [1911]. 1·9 g. of the phosphatide, which will be referred to henceforth as P1, were emulsified in 200 ml. water, and 200 ml. acetone were added. The precipitate P2, 0·5579 g., was removed by filtration; to the filtrate sodium chloride was added until further precipitation ceased. This precipitate P3 weighed 0·976 g. The analyses of P2 and P3 were so similar that they can be grouped together and compared with those of P1.

Table IV. *Recovery after precipitation of the phosphatide emulsion by acetone.*

	P2 and P3	P1	% recovery referred to P1
Yield % of P1	80·7	—	—
Ash	5·23	4·27	98·86
N	0·86	1·52	45·66
P	3·29	2·97	89·19
Fatty acids	63·2	57·0	91·18

Several deductions can be made from Table IV. In the first place the emulsification process has removed 54·34 % of the original nitrogen with a relatively small loss (10·81 %) of the phosphorus and has yielded a product having an N:P ratio of 1:2·61. Further, this mild treatment, which would largely remove water-soluble inorganic material, including inorganic phosphate, and which would not cause any appreciable hydrolysis of the phosphatide, has not removed the original inorganic constituents, for there is a virtually complete recovery of the ash on incineration. The N:P ratio and the nitrogen content (N 0·86 %) definitely show that the phosphatide mixture contains non-nitrogenous phosphatides, for not more than 50 % of lecithin (N 1·74 %) can be present. There is thus strong presumptive evidence for the presence of phosphatidic acid. 0·86 % N, calculated as lecithin, would require P 1·93 % and fatty acids 35·1 %, leaving 1·36 % P and 28·1 % of fatty acids to be accounted for. 1·36 % P would represent 33 % of the fraction if present as the alkaline earth salts of phosphatidic acid and would yield 25 % of fatty acids on hydrolysis. With the 6·2 % of unsaponifiable matter present in P1, 89 % of the fraction can be thus tentatively assigned. Thus this process has left in the product some 10 % of impurity. (Calculation also shows that the 19·3 % of material, removed as water-soluble products, has N 3·78 %, P 1·66 %, fatty acids 26·0 %. Such an amount of phosphorus would correspond to 43 % of it being present as lecithin or similar phosphatide, which would require 0·75 % N and would yield 30·2 % of fatty acids on hydrolysis.)

This experiment suggests therefore that the phosphatide mixture consists of lecithin and kephalin, together with about two-thirds of the sum of their amounts as salts of phosphatidic acid, and the method adopted provides an excellent means for concentrating the phosphatide with relatively little loss.

Before treating the main bulk of material, further preliminary work was carried out with a view to determining the best conditions for the isolation of phosphatidic acid. The original method used by Channon and Chibnall [1927] and also used by Chibnall and his co-workers was to shake the mixed phosphatides in ethereal solution with dilute hydrochloric acid. After washing the solution with water, the residue left after evaporation of the solvent was separated into a lecithin-kephalin fraction and phosphatidic acid by virtue of the solubility of the latter in acetone. The phosphatidic acid was then further purified by conversion into the lead salt. We encountered two difficulties when we attempted to carry out this process with our material. The first of these was the production of persistent emulsions when the mixed phosphatides were shaken in ethereal solution with aqueous acid, due to the lecithin and kephalin present. These were not encountered in the work on cabbage where the latter phosphatides were absent, but are commented on by Smith and Chibnall [1932] in their grass studies. This difficulty which rendered quantitative separations impossible, was overcome by a method which should prove of general use in phosphatide studies. We have used in place of water a mixture of water 100, acetone 40, sodium chloride 5. Acid may be added to the mixture as required and for final washing the salt may be omitted if desired. This has been found an extremely useful device, for separations are usually both rapid and quantitative.

The second difficulty was that we were unable to separate phosphatidic acid from the mixed acid-washed phosphatides by precipitation of the lecithin-kephalin fraction with acetone. Many variations in this procedure were attempted, but studies of the nitrogen and phosphorus percentages in a large number of fractions (an example is fraction P4 given later) showed that little separation was obtainable by this method. We have no suggestion to offer in explanation, save that perhaps the nature of the fatty acids present in the phosphatidic acid, or the position of attachment of the phosphoric acid to the glycerol molecule may be involved. The method adopted to overcome this difficulty has been to shake the phosphatide mixture, after washing with acid, directly with lead acetate. The lecithin fraction was then largely separated from the lead salts by repeated extraction with absolute alcohol. This process was only effective because the percentage of kephalin in our product was low.

In the fractionation which follows, the theoretical analytical figures below will serve for comparison with the results described. Any phosphatide preparation is a mixture of compounds varying in the nature of the fatty acids, and these calculated figures given are based on compounds containing two molecules of oleic acid.

	N	P	C	H	Mol. wt.	Fatty acids yielded by hydrolysis %	Ash as pyrophosphate %
Lecithin	1·74	3·86	65·75	10·71	803	70·3	—
Kephalin	1·88	4·17	66·08	10·48	743	75·9	—
Calcium phosphatidate	—	4·20	63·33	9·88	739	76·3	17·18
Lead phosphatidate	—	3·42	51·66	8·06	906	62·3	32·45
Magnesium phosphatidate	—	4·29	64·71	10·09	723	78·1	15·36
Potassium phosphatidate	—	4·00	60·31	9·15	775	72·7	21·28
Phosphatidic acid	—	4·42	66·76	10·41	701	80·5	—

Fractionation of the main preparation.

1. *Treatment by emulsification in water and precipitation by acetone.* 30·8 g. P1 (N, 1·52; P, 2·97 %) were emulsified in 500 ml. water and, after adding acetone (500 ml.) and 12·5 g. sodium chloride, the mixture was chilled for 15 hours. The precipitate P2, 27·1 g. (N, 0·94 %; P, 3·28 %), was treated in similar manner with half the quantities of water, salt and acetone. The precipitated phosphatide P3 weighed 25·38 g. (N, 0·87; P, 3·26 %). The first treatment thus removed 45·36 % of the nitrogen; the small amount removed by the second treatment, 7·28 %, was taken as evidence that further repetition of the procedure would not materially improve the product. The total loss of phosphorus into the aqueous phase was 11·5 %.

2. *Treatment with acid.* 25·1 g. P3 dissolved in 300 ml. ether were shaken successively with 300, 150 and 150 ml. of a mixture of water 400 ml., acetone 200 ml., sodium chloride 40 g., with sufficient hydrochloric acid added to make the solution *N*/20. The ethereal solution was washed five times with the acetone-brine solution without added acid, 700 ml. in all, and yielded on evaporation 24·4 g.

3. *Separation by ether-acetone.* 24·3 g. dissolved in ether (80 ml.) were precipitated with acetone (480 ml.). The process was repeated three times. The combined mother-liquors were evaporated to dryness and, by chilling the product after dissolution in hot acetone, a further precipitate was obtained. The total acetone-insoluble fraction P4 was 22·65 g.; the soluble fraction P5 weighed 1·64 g.

The yields and the analyses of these four products and that of P1 were:

	Yield g.	C %	H %	N %	P %
P1	30·8	60·88	9·56	1·52	2·97
P2	27·1	62·93	9·69	0·94	3·28
P3	25·38	60·42	9·31	0·87	3·26
P4	22·65	58·82	9·39	0·93	3·54
P5	1·64	71·25	11·32	0·25	1·53

4. *Treatment with lead acetate.* (*a*) *Acetone-soluble fraction P5.* 1·56 g. dissolved in 70 ml. ether were shaken with 20 ml. aqueous lead acetate solution containing a little acetic acid. The ethereal solution after four washings with water yielded 1·66 g. This was dissolved in 10 ml. ether and the lead salt precipitated by adding 30 ml. alcohol. The lead salt was again dissolved in ether and reprecipitated by alcohol. The twice precipitated lead salt P6 weighed 0·57 g. The soluble residue 1·09 g. was discarded.

(*b*) *Acetone-insoluble fraction P4.* In an attempt to avoid the emulsions encountered under (*a*), a saturated lead acetate solution in 50 % aqueous acetone was employed. 17·8 g. P4 dissolved in 180 ml. ether were shaken with 180 ml. of this lead acetate solution and the mixture was centrifuged. The ethereal solution was washed in the centrifuge-cups four times with 40 % aqueous acetone. On evaporation this yielded 19·5 g. while from the emulsion 2·43 g. were obtained; total yield 21·93 g. The product was extracted three times with hot absolute alcohol, 350 ml. in all, each extract being cooled to room temperature before filtration. The insoluble residue P8 weighed 7·80 g., the soluble fraction P9 12·2 g. The analyses of the lead compounds P6 and P8, together with the lecithin-kephalin fraction P9, were as follows:

	Yield g.	C %	H %	N %	P %	Pb %	Ash %
P6	0·57	52·02	7·74	0·12	3·25	21·6	30·7
P8	7·80	46·79	7·06	0·13	3·69	23·3	34·8
P9	12·20	62·18	10·11	1·30	2·45	—	2·2

The analytical figures for fractions P6 and P8 are in fair agreement with those required by lead phosphatidate, although these products are not pure. Our experience bears out that of Smith and Chibnall [1932]. These workers comment on the difficulty of obtaining reproducible figures for carbon and hydrogen on similar lead salts. Thus their crude lead phosphatidate preparation gave C, 50·3; 48·2 %; and on purification by precipitation by alcohol from a solution in ether these salts contained 47·0 to 48·3 % of carbon, as against their theoretical content of 51·2 %. Hence not too much reliance can be placed on these carbon figures, which are usually some units below the theoretical value. Our two fractions contain also about 0·1 % N, which we failed to remove in spite of various treatments attempted for this purpose. This same difficulty was experienced by Chibnall and Channon [1927] and Channon and Chibnall [1927], who were unable to remove the 0·11 % N from the free phosphatidic acid prepared from cabbage leaves. Further evidence that these products are mixtures was obtained in the preparation of phosphatidic acid from the lead salt. 0·39 g. P6 was suspended in 40 ml. of hot benzene and decomposed with hydrogen sulphide. Considerable difficulty was encountered in dealing with the colloidal solution which resulted. It was finally evaporated to dryness and the residue extracted with acetone. After filtration, the acetone solution was cooled to 0° for 24 hours and again filtered. On evaporation there was obtained 0·284 g. of a viscous oil, which represented 73 % of P6 as against the 78·4 % required by theory. This contained 3·9 % P (theoretical P 4·42 %) and had I.V. 108. Similar difficulties with colloidal solutions were encountered in the decomposition of 1 g. of fraction P8. This was treated similarly and, after standing in the ice-chest, the final acetone solution yielded after filtration 0·70 g. (P, 4·25 %; N, 0·15 %), whereas the theoretical requirement is 0·77 g. Further the mol. wt. of the product 718, determined by titration in alcohol, shows the presence of non-acidic impurities. Similar findings were recorded by Smith and Chibnall [1932] whose product had mol. wt. 746. This was confirmed by hydrolysis of 1·09 g. of P8 by boiling for 20 hours with 2·5 *N* hydrochloric acid. The yield of fatty acids so obtained, 0·542 g., was 49·6 % of P8 which is no more than 79·8 % of theory, an experience again similar to that of Smith and Chibnall, who obtained 77 % from the lead phosphatidate prepared from grass. These fatty acids had I.V. 107, and contained 24·4 % of saturated and 75·6 % of unsaturated acids determined by the Twitchell method. This I.V. is greater than that of the acids from the lecithin-kephalin fraction, 90·2, but no significance can be attached to this difference, because considerable oxidation of the acids must have taken place during the two years throughout which this work was being carried out, since the I.V. of the acids of the starting material was 120. It seems to us not improbable that the low yields of fatty acids from the lead salts is due in part to oxidation of the more unsaturated constituents and their consequent insolubility in light petroleum on liberation by hydrolysis. This might, in part, also explain the low carbon figures which are generally obtained in these preparations.

The lecithin-kephalin fraction P9.

The N : P ratio in this fraction was 1 : 0·9, and the low nitrogen and phosphorus values showed that not more than two-thirds of this fraction could be lecithin. The excess of nitrogen over phosphorus, together with the demonstration of the presence of carbohydrate, which is mentioned below, show that, in spite of the treatment by emulsification in water twice and shaking the ethereal solution with aqueous acid, this fraction still contains much non-lipoid material.

Calculation suggests that about 20 % of this fraction (3·1 g.) is not lipoid, which shows that the impurities still present after the double emulsification process, about 15 % of P3, have accumulated in this fraction. The difficulty of removing carbohydrates from plant phosphatide preparations has been encountered by many workers [Levene and Rolf, 1925; Schulze and Winterstein, 1903–04; Winterstein and Smolenski, 1908–09; Smolenski, 1908–09; Trier, 1913]. No attempt could be made to isolate lecithin or kephalin from so small an amount of material, and we accordingly contented ourselves with carrying out various experiments, designed to throw further light on the composition of this fraction.

(*a*) *Presence of carbohydrate.* Acid hydrolysis sufficiently prolonged to ensure the complete breakdown of the phosphatide resulted in the presence in the aqueous filtrate of reducing substances to the extent of 3 % when calculated as glucose. This contamination of plant phosphatide preparations with sugars is a general phenomenon, and Smith and Chibnall [1932] point out that the conditions used for acid hydrolysis of phosphatides are such as to reduce, possibly seriously, the yield of reducing sugar found. Hence this figure of 3 % is probably considerably too low and carbohydrates may thus account for a considerable part of the non-phosphatide material.

(*b*) *Fatty acid content.* By alkaline hydrolysis with alcoholic potassium hydroxide, the fraction was found to contain 6·8 % of unsaponifiable matter and 57·39 % of fatty acids. If present as lecithin, the phosphorus content would yield about 45 % of fatty acids on hydrolysis. The excess 12·0 % of fatty acids (about 1·5 g.) may have been produced by slight hydrolysis during the very many fractionation processes. The fatty acids had I.V. 90·2 and yielded by the Twitchell process 58 % of unsaturated acids and 42 % of saturated acids.

(*c*) *Amino-N content.* Determinations of total and amino-N on aliquot parts of the aqueous filtrate from an acid hydrolysis of 2 g. were carried out. The amino-N 0·30 % represented 25·7 % of the total N present in the hydrolysate, or 46·6 mg. which was the amount present in the original material P1. The remainder of this hydrolysate was used for the base separation.

(*d*) *Isolation of choline and colamine as gold salts.* The methods used by Smith and Chibnall [1932] were employed for the separation. The aliquot of the hydrolysate employed contained 3·53 mg. of amino-N and yielded 65·3 mg. of colamine chloroaurate, which was 65 % of that likely to be present from the amino-N content. It melted at 190° and contained 48·1 % Au (required 189–191°, Au 49·2 %). The filtrate from the mercuric acetate precipitation of the colamine was made acid and concentrated, and the mercury was removed as sulphide. After evaporation to dryness, the residue was dissolved in alcohol and an excess of alcoholic mercuric chloride added. The choline mercurichloride which crystallised was obtained in poor yield in very impure state. The mercury was therefore removed by means of hydrogen sulphide and after concentration of the filtrate a slight excess of gold chloride was added. The 10·29 mg. of non-amino-N present in the original aliquot gave 292 mg. of gold salt (M.P. 240–241°; Au 42·9 %). This product was equally impure, for choline chloroaurate melts at 269° and contains 44·5 % Au. From several preparations of the choline fraction of the phosphatide base, the highest melting-point for the gold salt was 248–249°.

Investigation of the inorganic constituents.

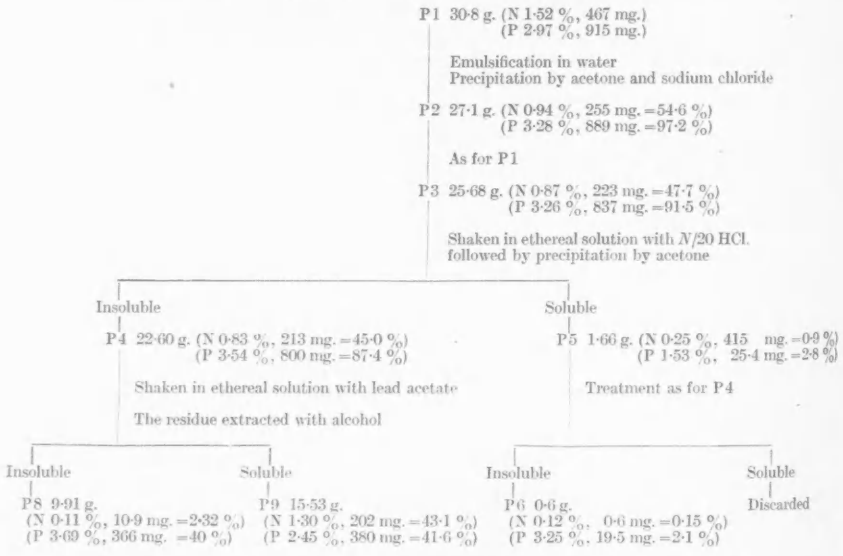
5·19 g. P1 were hydrolysed by boiling for 15 hours with *N* HCl. The fatty acids were removed by extraction with ether. The combined aqueous acid washings were concentrated to small volume and contaminating organic matter

removed by oxidising with nitric acid and perhydrol. Estimations of calcium as oxalate, magnesium colorimetrically and potassium as sulphate on suitable aliquot parts of the solution gave: Ca, 23·4 mg.; Mg, 28·4 mg.; K, 11·1 mg. The amounts of the metals found correspond to 59·22 mg. P, when calculated in ratios Ca/P, Mg/P, K/P, of 1, 1 and 2 respectively. If these metals were originally present as phosphatidates, the amounts in the 5·19 g. of P1 would be Ca phosphatidate, 0·432 g. (8·3 %): Mg phosphatidate, 0·8553 g. (16·5 %); K phosphatidate, 0·1103 g. (2·12 %). In the absence of other contaminating inorganic constituents these percentages of salts of phosphatidic acid would cause the original preparation to give an ash (as pyrophosphates) of 4·41 % (found 4·27 %).

Discussion.

For ease of discussion of the results, a summary of the fractionation is given in outline below. The percentages of N and P in each fraction are recorded, together with the total N and P contents, and these are expressed as a percentage of the corresponding figures on the original material. The weights of fractions have been corrected for the aliquots used for analysis.

Diagram 1. Balance sheet showing the corrected weights, nitrogen and phosphorus percentages and percentage recovery.


P1 30·8 g. (N 1·52 %, 467 mg.)
(P 2·97 %, 915 mg.)
Emulsification in water
Precipitation by acetone and sodium chloride
P2 27·1 g. (N 0·94 %, 255 mg. =54·6 %)
(P 3·28 %, 889 mg. =97·2 %)
As for P1
P3 25·68 g. (N 0·87 %, 223 mg. =47·7 %)
(P 3·26 %, 837 mg. =91·5 %)
Shaken in ethereal solution with N/20 HCl.
followed by precipitation by acetone
Insoluble
Soluble
P4 22·60 g. (N 0·83 %, 213 mg. =45·0 %)
(P 3·54 %, 800 mg. =87·4 %)
P5 1·66 g. (N 0·25 %, 415 mg. =0·9 %)
(P 1·53 %, 25·4 mg. =2·8 %)
Shaken in ethereal solution with lead acetate
Treatment as for P4
The residue extracted with alcohol
Insoluble
Soluble
Insoluble
Soluble
P8 9·91 g.
(N 0·11 %, 10·9 mg. =2·32 %)
(P 3·69 %, 366 mg. =40 %)
P9 15·53 g.
(N 1·30 %, 202 mg. =43·1 %)
(P 2·45 %, 380 mg. =41·6 %)
P6 0·6 g.
(N 0·12 %, 0·6 mg. =0·15 %)
(P 3·25 %, 19·5 mg. =2·1 %)
Discarded

In this discussion of the relative amounts of the various constituents present, the calculations will refer to the phosphorus content and not to the gross weight of the individual substances, as it appears more informative to discuss the distribution of the phosphatides on this basis. The diagram shows that the total lead phosphatidate isolated in fractions P6 and P8 contains 42·1 % of the total phosphorus originally present. Since choline and colamine were isolated as gold salts from the lecithin-kephalin fraction in amounts which compared favourably with the content of total and amino-N, it is reasonable to regard

the amino-N of this fraction as originating from kephalin, and to assess the remainder of the P after subtracting the kephalin-P as representing lecithin. Fraction P9 thus contains 2·48 g. kephalin and 7·2 g. lecithin, corresponding to 11·3 % and 30·4 % of the P of the original material. It remains to account for the phosphorus in the aqueous mother-liquors resulting from the emulsification of fractions P1 and P2. We made no attempt to investigate these further, save to show that the phosphorus present in them was not in inorganic form. Bearing in mind the impossibility of precipitating lecithin quantitatively by the means adopted, it is probable that the greater part of this phosphorus was present as lecithin. If this be assumed and the possibility that the original material contained substances containing phosphorus other than phosphatides be disregarded, a balance sheet to show the lipin-P distribution may be drawn up. We recognise of course the limited value of attempting to assess the proportions of different phosphatides in a mixture in the light of the limited knowledge of the constituents and the deficient methods of separation at present available, but such a distribution table is of some value in this particular instance, where attempts to maintain all separations as quantitative as possible have been made. On this basis, the total P of the original mixture had the distribution: lecithin, 46·6 %; kephalin, 11·3 %; phosphatidic acid, 42·1 %.

It remains to correlate these findings with the percentage of ash and its composition. It is shown in a preceding section that calculation of the amounts of the Ca, Mg and K in fraction P1 corresponded to a theoretical ash value of 4·41 % as against 4·27 % found if the metals were present as salts of phosphatidic acid. These amounts would account for 1·141 % of the P of P1, or 38·41 % of the total. The excellent agreement between this figure and that derived from the amount of crude phosphatidic acid, 42·1 %, must however be regarded with caution. In the first place the literature shows repeatedly that preparations of pure lecithin almost always give some figure for ash, and in our own experience two such preparations gave varying figures of 1 to 3 %. Our phosphatide preparation P1 with an ash of 4·27 % therefore was giving a relatively low value, and this percentage is no more than is required were its metallic constituents entirely present as phosphatidates. Secondly, it seems rather doubtful if the potassium were present as phosphatidate, for the alkali salts of phosphatidic acid are water-soluble. It must not be overlooked, however, that ethereal solutions of phosphatides will readily dissolve many substances which are insoluble in ether though soluble in water.

Previous work on the phosphatides of wheat germ has been confined to determinations of ether-soluble P or the recognition of the presence of lecithin from a study of the hydrolysis products. In regard to these and other findings, reference may be made to the work of Schulze and Steiger [1889], Winterstein and Hiestand [1907–08], Schulze [1908], Andrews and Bailey [1932], Sullivan and Near [1933], Knowles and Watkin [1932].

The function which salts of phosphatidic acid are playing in the plant cannot be assessed on the present limited evidence. In leaves, Channon and Chibnall found calcium phosphatidate to be the only phosphatide present in the case of the cabbage. Smith and Chibnall [1932], who encountered very great difficulty in extracting the phosphatides of grass, were finally compelled to use grass dried for 8 minutes at 200°. This material contained many phosphatide decomposition products but the investigations showed that in this leaf lecithin and kephalin were also present. Jordan and Chibnall [1933] obtained 0·4 g. of crude phosphatide from the embryo axes of 5000 seeds of the runner bean and, so far as could be achieved on this very limited amount of material, the results

suggested that the major part of the mixture consisted of a salt of phosphatidic acid, together with lecithin-kephalin in the proportions of 1:2. Our results on wheat germ give a corresponding ratio of 1:1·5. These authors found that on germination the disappearance of the cotyledon phosphatides was much more rapid than the appearance of phosphatides in the growing organs and hence they deduced that seed phosphatides must function chiefly as reserve food.

Summary.

1. Before fractionation of a preparation of the phosphatides of wheat germ, investigations were made of the best methods for extraction of the germ and of precipitation of the phosphatide from the extract.

2. Analyses of the oil prepared from seven samples of germ are recorded. The phosphatide fractions from these extracts were analysed for their contents of nitrogen, phosphorus and ash, together with the yields of fatty acids obtained by hydrolysis.

3. The deductions made from these analyses were confirmed by a detailed investigation of the phosphatide fraction prepared from 9·5 kg. of wheat germ. This fractionation showed the presence of phosphatidic acid (as calcium, magnesium and potassium salts), lecithin and kephalin. These substances occur in the approximate proportions of 4:4:1 when referred to the phosphorus content; at least 42 % of the total phosphatide being present as phosphatidic acid.

4. A method of working ethereal solutions of phosphatides with aqueous solutions, which largely overcomes the persistent emulsions usually resulting, is described.

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We wish to record our thanks to Messrs Bemax, Ltd., who supplied the fresh wheat germ.

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CXXII. THE RELATION OF THE GROWTH RESPONSE TO DOSAGE OF VITAMIN A.

CONFIRMATION OF A CURVE RELATING RESPONSE TO DOSE OF VITAMIN A GIVEN.

By KATHARINE HOPE COWARD.

From the Pharmacological Laboratories, Pharmaceutical Society of Great Britain, London.

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Coward *et al.* [1930] published a curve which related the average increase in weight in 5 weeks of groups of rats which, having ceased to grow on a diet deficient in vitamin A, had then been given graded doses of a certain sample of cod-liver oil. Each group consisted of about 30 rats (nearly equal numbers of males and females) and all the rats of any one group were given the same daily dose of cod-liver oil for 5 weeks. The different groups of rats were given daily doses of 0·25, 1·0, 1·5, 2·5, 7·5 and 20·0 mg. cod-liver oil respectively. The curve through the points obtained by plotting the mean increases in weight of the different groups against the dose of cod-liver oil given was a remarkably smooth one and apparently logarithmic in character. By plotting the mean increases in weight against the logs of the doses given, the points were found to be nearly in a straight line. The best straight line through these points was calculated, $y=12{\cdot}1+40{\cdot}2x$; in the form $y=12{\cdot}1+40{\cdot}2\log x$ it applied to the curve itself. Thus it follows that in a comparison of two substances the relative potency of the doses tested is not the ratio of the mean increases in weight of the groups of rats used; it is the ratio of the abscissae corresponding to the mean increases in weight in the curve relating mean increase in weight to dose of vitamin A given; for example if, in a simultaneous test of two samples of cod-liver oil, 1 mg. of one sample given daily for 5 weeks produced a mean increase in weight of 16 g. in a group of 5 male and 5 female rats, and 1 mg. of another sample of cod-liver oil produced a mean increase in weight of 8 g. in a similar group of rats, the ratio of the potencies of the two samples would not be $16:8=2$ (the ratio of the mean increases in weight) but $1{\cdot}25:0{\cdot}79=1{\cdot}58$ the ratio of the abscissae corresponding to the mean increases in weight as obtained from Coward's curve of response.

Use of this curve for comparison of two substances of different kinds.

The curve of response is needed for interpreting the mean increases in weight of two groups of rats which have been given doses of very different substances (*e.g.* cod-liver oil and, say, milk powder). It is therefore necessary to show that it holds good (*i.e.* measures only vitamin A), no matter what other factors may be unavoidably given to the rats in either or both of the doses of substance under test. That a curve of response may be unreliable and even useless has been shown by Coward *et al.* [1933] in work on the estimation of vitamin B_1. A rather shallow curve of response was obtained when vitamin B_2 *etc.* was

supplied as 8 % yeast autoclaved for 6 hours at 15 lbs. pressure in the basal diet, and the results from pairs of doses of such substances as wheat germ, dried yeast and a certain food product could not be interpreted by it. The result from the higher dose of each substance was much higher than any point on the curve. However, when a fresh curve of response was constructed from results obtained with 20 % autoclaved yeast in the basal diet, a steeper curve of response was obtained than before and the results which could not be interpreted by the first curve could be interpreted by the second. Obviously there had been insufficiency of some factor (B_2?) in the first basal diet so that the full effect of the doses of vitamin B_1 alone could not be produced, but the substances tested (wheat germ, dried yeast, *etc.*) had apparently made up the deficiency.

This experience and a similar experience in the construction of curves of response relating increase in weight and dose of vitamin D given [Coward *et al.*, 1932] made us examine all the results of our vitamin A tests in which we had tested two or more doses of any one substance. The results indicate that our curve of response for vitamin A holds good, no matter what may be the nature of the substance tested. This, in turn, indicates that the basal diet used in our vitamin A tests contains enough of all substances which are necessary for growth except vitamin A.

The examination of results from two doses of any one substance.

In each experiment in which we have tested two or more different doses of one substance for vitamin A, we have determined the apparent potencies of the doses tested by finding from the curve of response the abscissae corresponding to the mean increases in weight of the rats used for each dose. These give the apparent ratio of the doses, and by comparison of the apparent ratio with the known ratio some idea of the reliability of the curve of response may be obtained.

In practice we have seldom used the curve of response already described in this paper, for it requires an equal number of male and female rats in each group of animals used. In 1931 we separated the results from the male and female rats in the groups used for constructing the curve of response, and constructed other curves of response for male and female rats separately. The curves were $y = 11{\cdot}3 + 50{\cdot}3 \log x$ for the bucks and $y = 12{\cdot}4 + 27{\cdot}4 \log x$ for the does. Later, with more information from our tests, these equations became $y = 13{\cdot}4 + 45{\cdot}81 \log x$ (bucks) and $y = 12{\cdot}5 + 27{\cdot}0 \log x$ (does). Thus we now average separately the increases in weight of the bucks and does given any one dose, find the corresponding abscissae from their respective curves of response and find the weighted mean of these abscissae. This can then be compared with the figure similarly obtained from a group of rats on another dose.

In Table I are collected the results of all experiments in which two or more doses of a substance have been tested for vitamin A. In the last two columns are stated (*a*) the known ratio of the doses, and (*b*) the apparent ratio of the doses determined as just described. The average abscissae have been included in the table to show how wide a range of doses the curve may cover.

The results show very good agreement between the known ratio of the doses tested and the apparent ratio of the doses. All the tests but one (dried milk (*c*)) show better agreement than might be expected from the known error of the test. This is the more satisfactory when the varied nature of the products tested is realised. The products include butter and margarine (very good agreement), two samples of cod-liver oil (less good agreement, one rather high and the other rather low), a fish-meal (very good agreement), four samples of dried milk (two in good agreement, one too high and one too low and therefore not disturbing),

Table I. *Comparison between the known ratio of pairs of doses of different substances tested for vitamin A and the apparent ratio of the doses as determined from the abscissae of the curve of response corresponding to the mean increases in weight found in the tests.*

When the substance was given as a percentage of the diet, records were made daily of the amount of food eaten, and from these were calculated the average amount of test substance eaten per day throughout the experiment.

Substance	Dose	Weighted mean of abscissae for male and female rats	Known ratio of doses	Apparent ratio of doses, *i.e.* ratio of abscissae
Dairy butter	0·05 g. 0·1	1·34 3·36	2 : 1	2·5 : 1
Vitaminised margarine	0·05 g. 0·1	1·66 3·4	2 : 1	2 : 1
Cod-liver oil C	1 mg. 3 mg.	0·41 1·77	3 : 1	4·3 : 1
Cod-liver oil G	1 mg. 3 mg.	0·55 1·09	3 : 1	2 : 1
Fish-meal	2·5 % 25·0 %	2·37 25·6	10 : 1	10·8 : 1
(*a*) Dried milk (0·4 %) (2·0 %)	0·022 g. 0·15	0·42 5·05	7 : 1	12 : 1
(*b*) ,, (0·2 %) (2·0 %)	0·013 g. 0·155 g.	0·84 7·2	12 : 1	8·5 : 1
(*c*) ,,	0·1 g. 0·88 g.	3·2 12·0	9 : 1	3·8 : 1
(*d*) ,,	0·005 g. 0·04 g.	0·25 1·87	8 : 1	7·5 : 1
(*a*) Vitamin prep. 2 % 5 %	0·1 g. 0·36 g.	0·71 1·99	3·6 : 1	2·8 : 1
(*b*) ,,	0·01 g. 0·02 g.	1·3 3·4	2 : 1	2·6 : 1
Wheat embryo prep. 10 % 20 % 40 %	0·71 g. 1·56 g. 3·2 g.	0·64 1·74 3·86	4·5 : 2·2 : 1	6 : 2·7 : 1

two vitamin preparations (good agreement), a wheat embryo preparation (fairly good agreement). It is perhaps more satisfactory still when it is realised that some of the best results were obtained in tests where the ratios of the doses were greatest, *viz.* 10:1 and 8:1.

We therefore feel some confidence in using these curves of response for comparing mean increases in weight of groups of animals as treated in our vitamin A tests. The basal diet in use in vitamin A tests in this laboratory contains at least enough of each of the factors necessary for growth for the amount of vitamin A in the dose tested to exert its full effect.

The possible use of these curves in other laboratories.

Considering the influence of partial deficiencies of certain factors in the basal diet on the slopes of curves of response, it would be unwise to adopt the curves described in this paper in another laboratory without confirming them. The confirmation would not need as many rats as were used in the construction of these curves. Probably 10 male and 10 female rats on each of 3 doses, fairly widely distributed; for example 0·5, 2·0, 8 mg. of an average sample of cod-liver oil would suffice for this purpose.

Discussion.

We can find only one other report of the influence of the basal diet on the slope of the curve of response to graded doses of a vitamin. Sherman and Munsell [1925] published a series of composite growth curves of groups of rats which had been depleted of reserves of vitamin A and had then been given graded doses of tomato juice. Later Sherman and Batchelder [1931] published a series of composite growth curves of rats similarly prepared and then given graded doses of dried milk powder. They stated that "milk powder experiments showed a more accentuated response than doses of tomato to increasing levels of feeding." It is a simple matter to construct curves of response for 1, 2, 3, 4, 5, 6, 7 or 8 weeks' feeding from the figures of their two papers. We constructed the curves of response for 5 weeks' feeding, partly for comparison with our own curve, and partly because Sherman and Batchelder suspected that the rats of the most rapidly growing group suffered from a shortage of the "Coward factor" in the last week or two of the test. We could construct only one curve of response for each test, and not two separate ones for male and female rats respectively as the details for male and female rats were not given separately. Each group, however, consisted of approximately equal numbers of bucks and does. The curve of response to graded doses of dried milk powder was very much steeper than that to graded doses of tomato juice, and Sherman and Batchelder say "the less accentuated response to increasing levels of feeding of the tomato may have been because this was a less adequate source than milk of some factor or factors whose influence upon growth was not fully realised at the time of the earlier experiments, or possibly that, notwithstanding the general equivalence of the vitamin A of animal origin, and its precursor of vegetable origin, there may still be a difference in the degree of readiness and completeness of utilisation of the two forms." It is of interest to note that the basal diet used in Sherman's tomato experiment contained only 5 % of dried brewer's yeast, whereas the diet used in the milk experiment contained 10 % dried brewer's yeast. The greater "spread" of the results of the tests of graded doses of dried milk may have been due to the larger supply of some factor in the dried yeast or to some factor other than vitamin A supplied by the milk and not contained in the basal diet. Whichever was the cause, the results demonstrate afresh the necessity of using a basal diet which has been proved to contain sufficient of all factors necessary for growth except the one for which a test is to be made.

Morgan has constructed curves of response relating mean increase in weight in 5 weeks of rats which, having nearly ceased to grow on a diet similar to our vitamin A-free diet, have then been given graded doses of vitamin A (private communication). They are $y_1 = c_1 + 37{\cdot}6 \log x$ (bucks) and $y_2 = c_2 + 26{\cdot}1 \log x$ (does), but as his rats fed on this diet do not cease to grow on the basal diet alone, the slopes of the curves cannot fairly be compared with our own.

The following equations of the curves of response of similar tests for vitamin A, so far available for comparison, are therefore:

(*a*) Sherman's tomato juice curve, $y = 9{\cdot}9 + 22{\cdot}6 \log x$.
(*b*) Sherman's dried milk curve, $y = 5{\cdot}3 + 69{\cdot}8 \log x$.
(*c*) Coward's curve, $y = 12{\cdot}1 + 40{\cdot}2 \log x$.

It appears to be easy to explain the difference in slope of Sherman's two curves on the grounds of insufficiency of some factor in the basal diet, but it is difficult to see how this could be the reason for Coward's curve being less steep than Sherman's steeper one, for it has been shown that Coward's curve holds

good for graded doses of substances containing many different growth factors in addition to vitamin A. Does Sherman's very steep curve of response to graded doses of dried milk powder indicate that when rats have been fed on a diet partially deficient in one factor and wholly in another factor for several weeks and are then given abundance of the former and graded doses of the latter, the response will be greater than if the rats had had abundance of the first factor throughout the experiment? It is impossible to say if this is so from the experiments so far recorded. It can only be emphasised again that if a curve of response is to be used for determining relative potencies of doses, it is of the utmost importance that the basal diet should contain abundance of all factors necessary for growth except the one for which the test is being made.

SUMMARY.

The validity of Coward's curve of response relating mean increase in weight in 5 weeks to dose of vitamin A given has been confirmed by the method of comparing the known ratio of two doses of a substance tested with their apparent ratio as obtained from the abscissae of the curve of response corresponding to the mean increases in weight of the rats given the respective doses.

Twelve substances have been tested in this way. They included butter, vitaminised margarine, fish-meal, cod-liver oil, dried milk, wheat embryo, *etc.*

The agreement between known ratio of doses and apparent ratio of doses left no doubt that the curve was reliable, especially in view of the varied nature of the substances tested and the very large ratio of some of the pairs of doses used (*e.g.* 10:1, 8:1).

The range of doses which can be compared by the use of this curve extends from a dose which is just sufficient to maintain weight of rats which have ceased to grow on a diet deficient in vitamin A, to a dose which is 50 times as great. There is no "flattening" of the curve, and it is therefore concluded that there is no deficiency in the basal diet except vitamin A.

Comparison with curves of response from other laboratories has been made and differences in slopes of curves of response have been discussed.

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CXXIII. SIMPLIFICATION OF VITAMIN TESTS. THE EFFECT OF GIVING DOSES HALF-WEEKLY FOR VITAMIN A TESTS, AND ONCE ONLY FOR VITAMIN D TESTS, INSTEAD OF GIVING DOSES DAILY.

By KATHARINE HOPE COWARD and KATHLEEN MARY KEY.

From the Pharmacological Laboratory, Pharmaceutical Society of Great Britain, London.

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The most laborious part of the work involved in testing for vitamins is, perhaps, the giving of a daily dose of the standard or of the substance under test to the experimental animals. It occurred to us that a great saving of labour would be effected if the doses could be given at less frequent intervals. We therefore carried out two experiments:

1. On the effect of giving (*a*) daily doses, and (*b*) half-weekly doses in a 5 weeks' test for vitamin A (resumption of growth).

2. On the effect of giving (*a*) daily doses, and (*b*) the whole of the 10 days' dose on the first day of the 10 days' curative period in a vitamin D test.

Both experiments indicated that the giving of doses every day in tests for vitamins A and D is unnecessary; the doses can safely be given at longer intervals than 24 hours, though we have not attempted to determine the limits of these intervals.

Moreover, since adopting the simpler method of dosing, we have obtained enough figures to show that it is at least as accurate as the more laborious method of dosing every day.

We have not extended our investigation to the water-soluble vitamins. It is quite probable that, owing to the nature of their action in the animal body (so far as it is at present understood), the giving of doses at longer intervals than 24 hours would not have the same effect as giving the doses daily.

I. Vitamin A test.

A. *Comparison of effects of giving doses (a) daily and (b) half-weekly.*

The technique employed in this test was that described by Coward *et al.* [1930]. A cod-liver oil, chosen for examination by the two methods of dosing, was diluted with olive oil so that one drop of the dilution (19–21mg.) contained 1 mg. cod-liver oil. Some rats which had ceased to grow on a vitamin A-deficient diet were given one drop of this solution daily, others were given 3 drops on Mondays and 4 drops on Thursdays. The mean increases in weight of the bucks and does were calculated separately for each group (Table I).

Table I. *Mean increase in weight of groups of rats which had become steady in weight and were then given cod-liver oil (a) daily, or (b) half-weekly, the total dose for each rat being 7 mg. per week for 5 weeks.*

	No. of rats		Mean increase in weight in 5 weeks g.	
Method of dosage	♂	♀	♂	♀
Daily	5	6	19·6	14·3
Half-weekly	4	7	20·0	14·0

It is obvious that the method of dosing half-weekly has given the same result as the method of dosing daily.

B. *Validity of this result.*

The possibility that two results in our vitamin A tests although apparently equal, may in fact be different, has been worked out for groups of 10 animals. The larger the number of animals in each group, the greater is the probability that the results are indeed alike.

First were calculated the probabilities that any result with a group of rats would be greater or less than the true result by given amounts; *e.g.* Table II shows the calculation by which it was found that there was a 1/22 chance that 10 male rats would give a result either greater than 153·7 % or less than 65·1 % of the true value and that 10 female rats would give a result either greater than 179·8 % or less than 55·6 % of the true value.

Table II. *Calculation of the probability (1/22 chance) that the result of a vitamin A test (a) based on a group of 10 male rats would be greater than 153·7 or less than 65·1 % of the value found, and (b) based on a group of 10 female rats, would be greater than 179·8 or less than 55·6 % of the value found.*

	Bucks	Does
Standard deviation of the increase in 5 weeks of the rats of this colony in vitamin A tests	14·72	11·02
Curve of response relating mean increase in weight in 5 weeks to dose of vitamin A	$y = 11{\cdot}3 + 50{\cdot}3 \log x$	$y = 12{\cdot}4 + 27{\cdot}4 \log x$
Logarithmic variance of the test (Standard deviation/slope of curve of response)	$\lambda_1 = \frac{14{\cdot}72}{50{\cdot}3} = 0{\cdot}293$	$\lambda_2 = \frac{11{\cdot}02}{27{\cdot}4} = 0{\cdot}402$
Log. var. of mean when 10 rats are used in a test	$\frac{\lambda_1}{\sqrt{10}} = 0{\cdot}093$	$\frac{\lambda_2}{\sqrt{10}} = 0{\cdot}127$
Probability (1/22 chance) that a result with 10 rats would be:	*$2{\cdot}007 \times 0{\cdot}093$ $= 0{\cdot}1867$ $= \log 1{\cdot}5371$ greater than 153·7 % or less than 65·1 % of the true value	$2{\cdot}007 \times 0{\cdot}127$ $= 0{\cdot}2549$ $= \log 1{\cdot}7984$ greater than 179·8 % or less than 55·6 % of the true value

* Table of x, Fisher, *Statistical Methods for Research Workers*, p. 76.

In a test made with 5 bucks and 5 does, there is a 1/22 chance that the true value is either less than 60·3 % (average of 65·1 and 55·6) or greater than 166·7 (average of 153·7 and 179·8) of the value found. There is a 1/44 chance that it would be one or the other. In a comparison of 2 doses with 2 groups of rats (5 bucks and 5 does in each), there is a $1/44 \times 1/44$ chance that the true value of one dose is 60·3 % of the found value while the true value of the

other dose is 166·7 % of the found value, *i.e.* there is a 1/1936 chance that one value is really more than 166·7/60·3 = 2·76 times the other. This probability is very small indeed. Other probabilities are shown in Table III.

Table III. *Chances that the true ratio of two doses apparently equal may in fact be greater than 1:1.*

Probability	True ratio	Probability	True ratio
1 in 1936	2·76	1 in 144	2·02
1 in 900	2·57	1 in 36	1·63
1 in 400	2·30	1 in 16	1·41
1 in 256	2·18	1 in 4·4	1·03
1 in 196	2·10		

Thus there is only a 1 in 4·4 chance that either of two results which are apparently equal may be more than 3 % greater than the other. This is much smaller than the "probable error" (1 in 2 chance) which is the probability most often used for describing the accuracy of a biological test. There is a 1 in 16 chance of one result being more than 40 % greater than the other. This again is a small chance. We, therefore, felt justified in changing from daily doses to half-weekly doses for vitamin A tests.

The calculation just described was based on the assumption that the standard deviation of the increase in weight in 5 weeks of rats given half-weekly doses of vitamin A was the same as that of rats given daily doses of vitamin A. We have not enough figures to calculate the standard deviation of the increase in weight of rats given doses half-weekly for 5 weeks, but since adopting this method of dosing we have gained a certain amount of information from which we have calculated the standard deviation of the increase in weight of rats given doses half-weekly for 3 weeks. This figure can be compared with the standard deviation of the increase in weight of rats given doses daily for 3 weeks (Table IV).

Table IV. *Standard deviation of the increase in weight in 3 weeks of rats which have been given doses of vitamin A (a) half-weekly, (b) daily.*

Doses given		No. of tests	No. of rats	Σd^2	Average variance	Standard deviation
Half-weekly	Bucks	78	326	41,936	169·8	13·03
	Does	83	430	30,958	89·22	9·45
Daily	Bucks	184	1014	118,764	143·09	11·96
	Does	194	1187	95,616	96·29	9·81

Thus the standard deviation of the increase in weight in 3 weeks was slightly higher for the bucks and slightly lower for the does which were given doses of vitamin A half-weekly, than for the corresponding rats which were given doses daily. It was, therefore, reasonable to use the standard deviation of the increase in weight of rats given daily doses for 5 weeks for calculations relating to the increase in weight of rats given half-weekly doses for 5 weeks.

II. Vitamin D test.

A. *Comparison of effects of giving (a) one dose at the beginning of the 10 days' curative period, and (b) 1/10 of that dose daily for 10 days.*

We made a direct comparison of these two methods of dosing first of all by using the standard solution of vitamin D. This showed that the two methods gave the same result, but we made another comparison of the two methods

using cod-liver oil (our oil Z) lest the finding with the standard should not be applicable to cod-liver oil. This experiment also gave the same result by the two methods and we, therefore, felt justified in adopting the simpler method of dosing in all estimations of the vitamin D content of samples of cod-liver oil.

(*a*) *Standard solution of vitamin D.* 19 rats from 8 different litters (approximately half of each litter) which had received a rachitogenic diet (Steenbock's 2965) for 3½ weeks were each given 5 units of the International Standard solution of vitamin D in one drop (20 mg.) of solution. They were given the basal diet without addition for the following 10 days. 20 rats, the other halves of the same litters, having as nearly as possible the same initial weights (*i.e.* paired), were given 0·5 unit standard vitamin D in 20 mg. solution daily. Ten days after dosing began, all the animals were killed and their ulnae and radii were examined by the method in general use in this laboratory [Dyer, 1931; Key and Morgan, 1932]. The average degree of healing of the 19 rats which received 5 units of vitamin D in one dose was 3·00 and the average degree of healing of the 20 rats which received 0·5 unit daily was 3·20. Thus the two methods gave almost the same result.

(*b*) *Cod-liver oil.* 10 rats from 3 litters (half of each litter) were each given 20 mg. cod-liver oil in one dose on the first day of the curative period, and 10 rats, the other halves of the same litters, were each given 2 mg. of the same cod-liver oil for 10 days. The average degree of healing, determined as before, was found to be 2·0 for the animals receiving one 20 mg. dose and 1·7 for those receiving 2 mg. daily. Thus the two methods of dosing again gave almost the same result (Table V). Moreover, the method, which gave a slightly higher result in one experiment, gave a slightly lower result in the other. It, therefore, seemed justifiable to conclude that the two methods gave equal results.

(*c*) *Other substances.* Most substances other than liver oils and vitamin D concentrates contain so little vitamin D that large doses of the substance have to be given in order to produce an effect. Thus butters containing from about 0·5 to 2·0 units per g. have to be tested in doses of 0·5 g. per day. This is about as much butter as can be conveniently given to a rat at one time; hence we have continued to give doses of butters and of most other substances every day as before.

Table V. *Comparison of the effects of giving* (*a*) *the whole dose at the beginning of the 10 days' curative period, and* (*b*) *a daily dose for 10 days' curative period.*

	Average healing in groups of rats after	
	(*a*) Whole dose at beginning of 10 days' curative period	(*b*) Daily dose for 10 days' curative period
Irradiated ergosterol (5 units altogether)	3·0	3·2
Cod-liver oil (20 mg. altogether)	2·0	1·7

B. *Comparison of the two methods of dosing in vitamin D tests with regard to the accuracy obtainable in the result.*

When we began to give the whole of the 10 days' dose of oil or standard at the beginning of the curative period, we delivered the dose from a micrometer syringe, wiping the nozzle carefully before putting it sideways into the rat's mouth and then wiping it once or twice on the side of the rat's mouth on withdrawal, to make sure that the rat had received the whole of the very small dose.

The volumes of the doses to be delivered were calculated from the average specific gravity of cod-liver oil and of the diluted standard as determined in this laboratory.

When we had used this method for some months we gained the impression that we were obtaining more uniform results than we had previously. A calculation of the standard deviation of the amount of healing, based on observations of 334 rats, gives a standard deviation 0·716. The standard deviation obtained for tests in which doses were given daily was 0·783. The improvement in accuracy is thus very slight; it reduces the probable error of the result, when 20 rats are used in an estimation, from 9 to 8 %. We are, therefore, satisfied that the method of giving the whole dose at the beginning of the 10 days' curative period gives a result which is not less accurate than that obtained by the method of giving doses daily.

We have applied this result to the routine of our vitamin A tests also. Whereas we used to give each rat which was being used for a vitamin A test a dose of 4 units vitamin D in 20 mg. olive oil twice a week, nowadays we give 8 units of vitamin D in 20 mg. olive oil once a week. This is a saving of labour also.

Summary.

The same results were obtained in a vitamin A test on a certain sample of cod-liver oil by giving the required amount either as daily doses or as half-weekly doses.

A statistical calculation showed that the probability that these results are really equal and not only apparently so is very high. There is approximately a 77 % chance that the results are within 3 % of each other. There is a 94 % chance that they are within 41 % of each other.

A further calculation of the standard deviation of the increase in weight during the test, since this method of dosing was adopted in this laboratory, shows that the method gives results of approximately the same degree of accuracy as did the former method. The standard deviation of the results from the bucks was slightly greater, that from the does slightly less, than the corresponding figures obtained by the other method of dosing.

Practically equal results were also obtained in two vitamin D experiments ((*a*) using a certain sample of cod-liver oil, and (*b*) using the International Standard for vitamin D) when the whole dose was given at the beginning of the 10 days' curative period and when the amount was divided into 10 daily doses. The former of these methods of dosing appeared by calculation to be slightly more accurate than the latter.

A great saving of labour in tests for vitamins A and D can be safely effected by giving doses at less frequent intervals than the usual one of 24 hours. There is no evidence yet that this result is applicable to tests on the water-soluble vitamins.

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CXXIV. THE INFLUENCE OF THE SOLVENT ON THE VITAMIN A ACTIVITY OF (*a*) CAROTENE AND (*b*) COD-LIVER OIL.

By FREDERICK JOHN DYER, KATHLEEN MARY KEY and KATHARINE HOPE COWARD.

From the Pharmacological Laboratories, Pharmaceutical Society of Great Britain, London.

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In June, 1931, the Permanent Commission on Biological Standardisation of the Health Organisation of the League of Nations recommended [1931] the adoption of a particular sample of carotene as a provisional standard of reference for the estimation of vitamin A. This was to be prepared from the pooled contributions of five workers from different laboratories by the National Institute for Medical Research, London, where it would be kept and issued as required. The unit of vitamin A suggested was the vitamin A activity of 1γ (0·001 mg.) of this particular sample of carotene.

The standard of reference for vitamin A was adopted provisionally for two years, for it was not known how long carotene would retain its growth-promoting potency. As soon as the standard became available, workers in many laboratories began to use it and, from informal comparisons of results, it early became evident that widely different responses to the same dose of standard were being obtained in different laboratories. It was difficult to believe that the differences in response were entirely due to differences in basal diets of the rats, and it seemed most probable that the cause of the differences would be found in the different oils used for dissolving the carotene for administration to the rats. It was, therefore, thought desirable to make direct comparisons of different oils.

I. The influence of the solvent on the vitamin A activity of carotene.

A. *First comparison of oils as solvents for carotene.*

In the first comparison the standard carotene was dissolved in (*a*) hardened cotton-seed oil, (*b*) ethyl laurate (redistilled), (*c*) arachis oil, respectively, so that a daily dose of 5γ carotene could conveniently be given in 0·02 g. oil. In preparing the solutions, one of two methods was adopted; either (*a*) the ampoule containing the standard was weighed before and after washing out with benzene and the loss taken as the weight of carotene, or (*b*) after transferring the solution to a known weight of melted solvent, the benzene was evaporated *in vacuo* until constant weight was attained and the increase in weight recorded as the weight of carotene added. Solvent was then added to produce the required dilution. The solutions were distributed in amber-coloured glass ampoules which were twice evacuated, refilled with dry nitrogen and immediately sealed. The tests were carried out by our usual method of giving 3 doses on Tuesdays and

4 on Fridays [Coward and Key, 1934]. A fresh ampoule of solution was opened each day on which doses were given. The results of this comparison are seen in Table I. All ampoules of solutions were kept at 0°.

Table I. *Comparison of (a) hardened cotton-seed oil, (b) ethyl laurate and (c) arachis oil as solvents for carotene for use in vitamin tests.*

Dose: 5γ carotene (International Standard) in 0·02 g. oil per day.

Solvent	No. of rats in test		No. of rats surviving		Mean increase in wt. in 5 weeks g.		Abscissae of Coward's curve of response corresponding to mean increase in wt.		Weighted mean of abscissae, *i.e.* relative potency of doses
	♂	♀	♂	♀	♂	♀	♂	♀	
Hardened cotton-seed oil	7	7	2	6	8·5	17·2	0·88	1·47	1·32
Ethyl laurate	5	6	4	6	9·2	22·0	0·91	2·24	1·71
Arachis oil	5	7	5	7	38·6	40·7	3·49	10·79	7·75

Thus the vitamin A value of 5γ carotene dissolved in arachis oil may be five or six times as great as it is when dissolved in hardened cotton-seed oil or in ethyl laurate.

The three oils (without carotene) were tested on 3 groups of rats, 4 rats in each group, each rat being given a dose of 0·02 g. oil per day (3 doses on Tuesdays and 4 on Fridays). One rat given hardened cotton-seed oil lived for 35 days and was then killed; it was 5 g. less in weight than when first given the oil; the other three died in 11, 19 and 27 days respectively. The rats given ethyl laurate died in 6, 7, 17 and 17 days respectively. The rats given arachis oil died in 14, 17, 21 and 35 days respectively. Thus the superiority of the arachis oil solution of carotene cannot be attributed to the presence of vitamin A in the arachis oil.

Some comparisons were made of the yellow and red values of these solutions (5γ carotene in 0·02 g. oil), (*a*) after the ampoules had been kept unopened in the refrigerator for 11 weeks after sealing, and (*b*) after a further week during which the open ampoule had been exposed to the air and warm temperature of the rat room for 20–30 minutes each day. The result may be seen in Table II. The colours were matched against the glasses of a Lovibond tintometer.

Table II. *Colours of solutions (a) after 11 weeks' storage in nitrogen-filled ampoules in refrigerator, and (b) same solutions after exposure to air and warm temperature of rat room for 20–30 minutes daily for one week.*

	Colour of solution from freshly opened ampoule after storage for 11 weeks		Colour of same solution after exposure to air of rat room	
	Yellow	Red	Yellow	Red
Hardened cotton-seed oil	19·6	7·0	20·3	6·4
Ethyl laurate	23·0	3·9	24·0	4·0
Arachis oil	24·0	5·7	24·0	5·7

Thus the colour of the solution seems to be very little indication of its vitamin A activity, for the solution in the hardened cotton-seed oil was only slightly less yellow and was a deeper red than the solution in arachis oil, and the solution in ethyl laurate had the same yellow value and a somewhat lower

red value than the solution in arachis oil. The exposure to the air and warm temperature of the rat room for 20–30 minutes daily for one week did not reduce the colour values of these solutions.

B. *Second comparison of oils as solvents for carotene.*

The second experiment consisted of a comparison of arachis oil and coconut oil as solvents for the Internationl Standard carotene. A private communication from one worker that the activity of carotene in arachis oil had been found to be much less than in coconut oil made us decide to use a dose of 2γ standard in arachis oil and a dose of 1γ standard in coconut oil for this comparison. One ampoule of the standard was used for each solution and the dilution made by the same method as in the last experiment. Three doses were given to the rats on Tuesdays and 4 on Fridays as before, a fresh ampoule of solution being opened each day (Table III).

Table III. *Comparison between (a) arachis oil and (b) coconut oil (161441) as solvents for the standard carotene.*

Solvent	Dose of standard	No. of rats used in the test ♂	No. of rats used in the test ♀	Mean increase in wt. in 5 weeks g. ♂	Mean increase in wt. in 5 weeks g. ♀	Abscissae of Coward's curve of response corresponding to mean increase in wt. ♂	Abscissae of Coward's curve of response corresponding to mean increase in wt. ♀	Weighted mean of abscissae, *i.e.* relative potency of doses
Arachis oil	2γ	4	5	33·5	23·6	2·75	2·56	2·64
Coconut oil (161441)	1γ	5	5	25·0	12·6	1·79	1·00	1·40

The relative potency of the doses as determined from the abscissae corresponding to the mean increases in weight of the groups of rats in Coward's curve relating increase in weight to dose of vitamin A given is 2·64:1·4 which is almost the ratio of the doses of carotene given, 2:1. Therefore, the activity of the standard in these samples of arachis oil and coconut oil (161441) is the same.

C. *Third comparison of oils as solvents for carotene.*

A third experiment was carried out (*a*) to compare different samples of coconut oil and (*b*) to see whether the addition of quinol to the solution would make any appreciable difference to the activity of the carotene within the duration of the experiment[1]. The British Drug Houses, Ltd.. kindly supplied three samples of coconut oil known to be of different origin. The test was made in the usual way on each of these samples, each one being tested with and without 0·1 % quinol respectively. The solutions were made in the usual way, one ampoule being opened on each day of dosing. The dose was at the rate of 1γ carotene per day (Table IV).

It is evident that the addition of quinol has made no difference to the activity of the carotene dissolved in each of these samples of coconut oil. In two of the samples the activity of the carotene appears to be equal, while in the third it appears to be less than in the other two. In order to determine whether this difference is significant a calculation was made on the basis of the increases in weight of (*a*) the bucks which received each oil, whether with or without quinol, and (*b*) the does which received each oil whether with or without

[1] A comparison suggested by the Accessory Food Factors Committee of the Medical Research Council and Lister Institute and undertaken by three laboratories.

Table IV. *Comparison between three samples of coconut oil, each one with and without 0·1 % quinol respectively, as solvents for carotene.*

Solvent	No. of rats used in test ♂	No. of rats used in test ♀	Mean increase in wt. in 5 weeks g. ♂	Mean increase in wt. in 5 weeks g. ♀	Abscissae of Coward's curve of response corresponding to mean increase in wt. ♂	Abscissae of Coward's curve of response corresponding to mean increase in wt. ♀	Weighted mean of abscissae, *i.e.* relative potency of doses	
C.O. 161441 + quinol	5	7	21·8	21·7	1·62	2·19	1·95	Av. 1·8
C.O. 161441 alone	3	8	16·7	19·3	1·28	1·79	1·65	
C.O. 162398 + quinol	3	7	28·3	17·7	2·18	1·56	1·75	Av. 2·1
C.O. 162398 alone	6	7	30·5	23·3	2·41	2·5	2·46	
C.O. 162750 + quinol	4	8	7·2	15·3	0·83	1·28	1·13	Av. 1·1
C.O. 162750 alone	3	7	−2·7	16·4	0·53	1·40	1·08	

quinol. The difference in mean increase in weight of the groups of bucks receiving oils 162398 and 162750 respectively was found to be significant, P (the probability that a correlation should arise by random sampling from an uncorrelated population) being $<0·01$. When, however, the calculation was made not from the increase in weight of each rat, but from the doses corresponding to the individual increases in weight (found from Coward's curve of response) P was $>0·05$ and $<0·1$. Therefore the difference indicated by the male rats is of doubtful significance. For the female rats P lies between 0·2 and 0·3 as calculated by the former method and by the latter method P is $>0·02$ and $<0·05$. The difference indicated by the female rats is therefore insignificant. The latter method must be accepted as the more accurate as the response (increase in weight) is proportional to the log of the dose given, not to the dose itself. When the results from both male and female rats are calculated as one result by the use of the abscissae of the curve only (not the increase in weight), P becomes very slightly less than 0·02. Thus there is some indication that there is a difference between the two samples of coconut oil but these results cannot be considered to have proved it.

II. The influence of the solvent on the vitamin A activity of cod-liver oil.

When the early experiments indicated that the activity of carotene was influenced by the oil in which it was dissolved, it appeared to be possible that the oil with which a sample of cod-liver oil was diluted for a test would influence the vitamin A activity of the cod-liver oil. This was investigated, using our cod-liver oil Z for the tests.

A. *Comparison of oils as diluents for cod-liver oil.*

The cod-liver oil, Z, was diluted so that the solution contained the daily dose of 1 mg. in 0·02 g. oil ($\pm 0·001$ g.). Fresh dilutions were made once a week during the test. The solvents tested were (*a*) hardened cotton-seed oil (the sample already used for the carotene experiments), (*b*) olive oil (sample bought in the open market) and (*c*) coconut oil (sample 161441 from B.D.H.). A simultaneous test of 2γ carotene (International Standard) in the same sample of coconut oil was also made (Table V).

Thus coconut oil may enhance the apparent vitamin A value of cod-liver oil or, alternatively, hardened cotton-seed oil and olive oil may lessen the apparent

Table V. *Comparison of (a) hardened cotton-seed oil, (b) olive oil and (c) coconut oil (161441) as diluents for cod-liver oil in a vitamin A test; and also a simultaneous test of the International Standard for vitamin A in coconut oil (161441).*

Dose (daily)	Solvent	No. of rats ♂	No. of rats ♀	Mean increase in wt. in 5 weeks g. ♂	Mean increase in wt. in 5 weeks g. ♀	Abscissae of Coward's curve of response corresponding to mean increase in wt. ♂	Abscissae of Coward's curve of response corresponding to mean increase in wt. ♀	Weighted mean of abscissae, *i.e.* relative potency of doses
1 mg. cod-liver oil Z	Hardened cotton-seed oil	3	7	1·0	13·1	0·62	1·06	0·93
,,	Olive oil	4	7	12·5	15·7	1·06	1·32	1·23
,,	Coconut oil (161441)	5	7	17·8	30·9	1·35	4·73	3·32
2γ carotene (Int. St.)	Coconut oil (161441)	5	7	37·6	33·7	3·33	5·99	4·88

vitamin A value of cod-liver oil. The significance of the difference between the results for olive oil and coconut oil is expressed by the value found for P, $<0{\cdot}01$.

A simultaneous test of the coconut oil (161441) on 6 rats 0·02 g. per day resulted in the death of five of the rats within 3–28 days. One rat was alive at the end of 5 weeks and had gained 15 g. Thus the coconut oil cannot be suspected of itself containing vitamin A.

B. *Estimation of the vitamin A potency of a sample of cod-liver oil (Z).*

(*a*) A test of a daily dose of 2γ carotene (International Standard) in the same sample of coconut oil (Table V) was made at the same time as the comparison of different oils as solvents for cod-liver oil, in order to determine directly the value of our cod-liver oil Z. It proved to be 1400 International Units per g.

(*b*) At the time of the test summarised in Table III, a simultaneous test was made of cod-liver oil Z. The daily dose was 1 mg. dissolved in 0·02 ($\pm$0·001) g. arachis oil. Fresh dilutions were (as usual) made each week (Table VI). From this comparison, cod-liver oil Z was found to contain 1750 International Units vitamin A per g.

Table VI.

1. *Estimation of the vitamin A content of cod-liver oil Z, the oil and the standard both dissolved in arachis oil.*

2. *Comparison of the vitamin A activity of a sample of β-carotene with that of the International Standard, both dissolved in coconut oil (161441).*

Dose	Solvent	No. of rats ♂	No. of rats ♀	Mean increase in wt. in 5 weeks g. ♂	Mean increase in wt. in 5 weeks g. ♀	Abscissae of Coward's curve of response corresponding to mean increase in wt. ♂	Abscissae of Coward's curve of response corresponding to mean increase in wt. ♀	Weighted mean of abscissae, *i.e.* relative potency of doses
1 mg. cod-liver oil Z	Arachis oil	5	5	25·2	24·8	1·82	2·84	2·33
2γ carotene (Inter. St.)	Arachis oil	4	5	33·5	23·6	2·76	2·56	2·64
1γ carotene (Inter. St.)	Coconut oil (161441)	5	5	25·0	12·6	1·79	1·00	1·40
1γ β-carotene	Coconut oil (161441)	5	5	30·0	23·6	2·36	2·56	2·44

There is no significant difference between the results (*a*) and (*b*). Thus the same estimate of the vitamin A potency of a sample of cod-liver oil was obtained by dissolving both oil and standard in either arachis oil or in coconut oil.

C. *Estimation of the vitamin A potency of a sample of β-carotene.*

Part of a sample of β-carotene kindly supplied to the Medical Research Council by Prof. Karrer was compared with the International Standard for vitamin A, both samples of carotene being dissolved in coconut oil, distributed in ampoules and used for feeding as in the previous tests. The result may be seen in Table VI. The β-carotene has about 175 % of the vitamin A potency of the International Standard.

Discussion.

Baumann and Steenbock [1933] have examined the influence of various solvents on carotene with respect to changes in colour intensity of the solutions. By measuring spectrophotometrically the intensity of $E^{1\%}_{1\,\mathrm{cm.}}$ 485 and 460$m\mu$, they found that, among edible oils, refined cotton-seed oil was outstanding as a stabilising solvent for carotene; esters of lauric and sebacic acids alone were unsatisfactory, but with quinol were as effective as cotton-seed oil. Although reduced by exposure to light, air and high temperature, solutions of carotene in cotton-seed oil were not rendered more stable by exclusion of oxygen or presence of quinol or the antioxidant factor of wheat germ oil. Under the conditions usually obtaining during feeding tests, carotene in cotton-seed oil suffered 26 % loss in the first month. Baumann and Steenbock recommend that the main supply of solution should never be removed from the ice-chest, but that a portion necessary for a week's feeding only should be removed for the short time while doses are given to the animals. Actually this is the procedure that we have always followed in our feeding tests with carotene and vitamin concentrates.

While we have confined our observations almost wholly to the biological activity of our solutions, our one physical comparison of different oils, hardened cotton-seed oil, ethyl laurate and arachis oil, indicated that, after 11 weeks, there was very little difference in the intensities of yellow colour of the solutions, although, during that period, biological tests had shown the solution in arachis oil to be more active than either of the other two solutions. Moreover, exposure of the solutions to the air and warm temperature of the animal room for 20–30 minutes daily for one week did not diminish the colour intensity of the solutions. Thus, while we have demonstrated biologically that different oils used in dissolving carotene for administration to the rats may influence greatly the activity of the carotene, our results with the physical measurements, so far as they go, do not indicate that the intensity of colour of the solutions can be taken as an indication of their vitamin A potency.

Summary.

1. The oil used for solution of the provisional International Standard for vitamin A may greatly influence its biological activity as shown in ordinary vitamin A tests.

2. A solution of the Standard in arachis oil had 5–6 times the potency of solutions in hardened cotton-seed oil and ethyl laurate, though made of the same strength, *w/v*.

3. Solutions of equal strength of the Standard in one sample of arachis oil and in coconut oil (161441) had the same vitamin A potency.

4. Two samples of coconut oil gave solutions of the same potency. A third sample appeared to give a solution of less potency but the result was not conclusive proof of a difference.

5. The oil used to dilute cod-liver oil for dosing may have some influence on the vitamin A potency of cod-liver oil also. Cod-liver oil Z dissolved in coconut oil (161441) was about three times as active as when dissolved in hardened cotton-seed or olive oil. The coconut oil itself (161441) was shown to be without vitamin A activity.

6. When cod-liver oil Z and the Standard were both dissolved in arachis oil for testing, the same estimate of the vitamin A content of oil Z was obtained as when both were dissolved in coconut oil.

7. An estimation of the vitamin A potency of a sample of β-carotene showed it to have about 175 % of the vitamin A potency of the International Standard.

8. The intensities of the yellow colour of different solutions of the Standard did not correspond with the vitamin A activities of the solutions but this comparison was made in only one experiment.

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CXXV. OBSERVATIONS ON THE CHEMISTRY OF LIVER GLYCOGEN.

By DAVID JAMES BELL[1] and FRANK GEORGE YOUNG[2].

From the Physiological Laboratory, Marischal College, the University of Aberdeen.

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The means of identification of glycogen at the investigator's disposal are of necessity limited by the physical nature of the material; indeed the identity of glycogens prepared from different sources has never been satisfactorily established. Cremer [1894] and Clautriau [1895] found values for the specific rotation of yeast glycogen of +198·9 and +184·5° respectively. Harden and Young [1902; 1912] found values between +191 and +203° for glycogens prepared from oysters, rabbit-liver and yeast; in addition, the colour developed with iodine varied in tint according to the source. Norris [1913] followed the course of hydrolysis of a number of specimens of glycogen by pig pancreatic amylase and observed that his materials were hydrolysed at different rates when the optimum p_H of the reaction was taken into consideration.

The recent work of Donhoffer and Macleod [1932] has suggested that the glycogen in the livers of fasted rabbits may differ in its physiological behaviour from that in the livers of fed animals, and it is of interest to know whether this physiological differentiation is associated with a difference in chemical properties. We have therefore endeavoured by carefully controlled methods to determine satisfactory criteria for a standard type of glycogen derived from the livers of fed rabbits and then to apply our data to specimens obtained under some different physiological conditions and from various sources. This paper is concerned solely with glycogens obtained from livers.

The criteria which we finally adopted were:

(1) Specific rotation in water.

(2) Reduction of the Hagedorn-Jensen ferricyanide reagent.

(3) Colour developed with dilute iodine solution, and opalescence of solution.

(4) Ash content.

(5) Polarimetric observations during the course of hydrolysis by dilute mineral acid.

Glycogen from fed rabbits.

Isolation.

The methods available fall into two types, *viz.* those in which the glycogen is extracted from the tissues by water and those in which the tissues are completely dissolved by strong alkali. Pflüger [1904] and the earlier workers concluded, with the methods then available, that glycogen was not affected by treatment with hot 30 % KOH solution, but this has been recently questioned; for example Haworth and Percival [1932] find that glycogen has a lower reducing power towards hypoiodite than they would infer from the properties of their

[1] Carnegie Teaching Fellow. [2] Beit Memorial Fellow.

postulated structural formula. They suggest that the reducing groups originally present in the glycogen molecules have been partly oxidised during treatment with strong alkali. We have therefore prepared, by methods of both types, *i.e.* aqueous extraction and alkali-extraction, a number of specimens of glycogen from the livers of fed rabbits, these specimens being subsequently subjected to examination under parallel conditions.

"Aqueous extracted glycogen" was obtained by the following method, which was essentially similar to that described by Carruthers [1931]. The livers, removed whole from three to five decapitated animals, were immediately plunged into about twice their volume of boiling water and the whole was heated to boiling for 5 minutes during which time the tissue was chopped into small pieces. The solid was then ground with sand and boiled with the original liquors for 30 minutes; it was then strained off and twice re-extracted with boiling water for 30 minutes. The united extracts were cooled, 40 % trichloroacetic acid solution was added until the final concentration was 4 %, and the whole was allowed to stand at 0° for 15–18 hours. The precipitated protein was centrifuged off and the glycogen precipitated by alcohol (2·5 vol.). The crude material was twice reprecipitated from water by alcohol in the presence of a little ammonium acetate as recommended by MacDowell [1927], when the glycogen separated as a pure white powder.

The "alkali-extracted glycogen" was obtained by direct alcoholic precipitation of the alkaline tissue hydrolysate from the action of 30 % KOH solution for 3 hours at 100°, using 3 to 5 livers for each specimen. As before, the crude product was twice reprecipitated from water by alcohol in the presence of ammonium acetate, but here the material invariably showed a faint brownish tinge.

As a final process of purification all the specimens were dialysed for 100 hours in running water in the presence of thymol. Dialysis was effected in collodion tubes prepared according to the directions of Wright *et al.* [1928]. The foregoing procedure yielded products which were nitrogen-free and did not reduce Fehling's solution.

In order to investigate the specific rotation, reducing power, *etc.*, the material was dried over calcium chloride at 100°/0·1 mm. until constant weight was attained. This required about 2 hours. No "denaturation" such as was observed by Sayhun and Alsberg [1930] took place during the drying of our specimens.

Table I.

Specimen	$[\alpha]_D^{18°}$ $c=0·5$ $l=1·0$	Reduction (glucose = 100)	
Alkali-extracted			
1	+191·5°	3·30	
2	199·0	0·24	
3	191·0	1·94	
4	192·7	0·08	
5	193·5	0·15	
	Mean	1·14	Iodine coloration (deep brownish red) and opacities were identical
Water-extracted			
6	192·7	0·21	
7	191·0	0·40	
8	191·0	0·37	
9	194·1	0·60	
10	199·0	0·27	
11	195·8	0·09	
	Mean	0·32	

Solutions in water, of concentration approximately 1·3 %, were prepared and portions diluted to 0·5 % for observations on rotation and reduction.

The results are given in Table I.

From the foregoing results it will be seen that the observed specific rotations of our specimens varied between the limits +191·0 and +199·0°, in both types of material. The alkali-extracted specimens showed considerable irregularity in reducing power, the average value being considerably greater than that of those prepared by aqueous extraction.

Hydrolysis of liver glycogen by aqueous acid.

The polarimetric behaviour on hydrolysis by aqueous acid was then investigated. To suitable amounts of the 1·3 % solutions small quantities of concentrated hydrochloric acid were added, sufficient to give a final concentration of 2·4 % HCl. The initial rotation was observed in a 1 dm. tube, and then the solution was heated at 100°. Observations were made at frequent intervals, using a 2 dm. tube; it was noticed that with both types of preparation the solution became clear after about 15 minutes, but while the solutions from the aqueous extracted material, when clear, were absolutely colourless, those of the alkali-extracted preparations showed a distinctly yellow tinge. On plotting rotation-time curves, a striking difference emerged between the curves derived from the two groups of specimens. Whereas the water-extracted material showed a perfectly regular and smooth curve in each case (Fig. 1), the alkali-extracted specimens, without exception, displayed varying, yet considerable, inflections during the first hour of hydrolysis (*e.g.* Fig. 2).

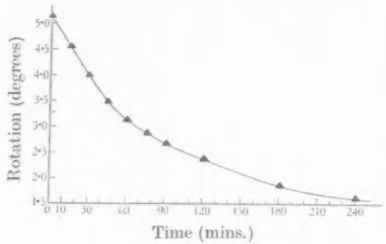

Fig. 1.

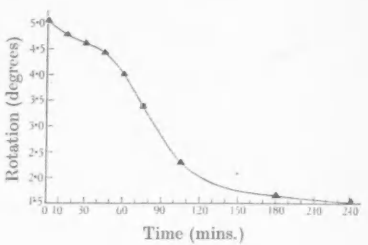

Fig. 2.

Fig. 1. Hydrolysis curve of specimen 8 (prepared by water process). Concentration of glycogen, 1·3 %.

Fig. 2. Hydrolysis curve of specimen 2 (prepared by KOH process). Concentration of glycogen, 1·3 %.

The effect of further treatment with KOH.

Specimens 6 to 10 inclusive (see Table I) were united and reprecipitated from water by alcohol to form specimen A. Amounts of this material, and also quantities of specimens 2 and 3, were heated as 5 % solutions in 30 % KOH for 2 hours, precipitated and dialysed as before.

No change in iodine coloration or opacity could be detected and no loss in material occurred. The results are given in Table II.

Table II.

Specimen	$[\alpha]_D^{18°}$, $c=0{\cdot}5$, $l=1$		Reduction	
	Before KOH	After KOH	Before KOH	After KOH
2	+199·0	+200·0	0·24	0·29
3	191·0	198·4	1·90	0·46
A	195·8	198·1	0·09	0·10

No significant change had occurred in the specific rotation, while the reducing power was considerably diminished where it had been initially large (specimen 3).

As before on hydrolysis by aqueous acid (2) and (3) showed marked inflection of the rotation-time curves, while (A), which had previously given a smooth hydrolysis curve, now exhibited an inflected one (Fig. 2).

The effect of precipitation from water by acetic acid.

Claude Bernard [1877] stated that glycogen could be precipitated from water by the addition of acetic acid, an observation which does not seem to have attracted the attention of later workers.

We have found that glycogen is precipitated quantitatively in 80 % acetic acid solution and have applied this to the estimation of glycogen in tissue, without, however, finding any particular advantage associated with its use in this connection. Nevertheless, precipitation with 80 % acetic acid provides an excellent means of freeing glycogen from the inorganic material with which it is normally associated. Thus we have found that, employing this process, it is possible after four precipitations to obtain glycogen with an exceedingly small ash-content, and in some instances we have prepared ash-free specimens, without having recourse to elaborate methods of electro-dialysis, *etc.* Table III gives some typical results:

Table III.

Specimen	$[\alpha]_D^{18°}$		Reduction		Ash content %	
	Initial	After pptn. by acetic acid	Initial	After pptn.	Initial	After pptn.
(KOH)						
2	+199·0°	+196·7°	0·24	0·11	—	0·195
3	191·0	190·7	1·90	0·06	—	0·224
P	—	196·9	—	0·09	—	0·090
(Water)						
A	195·8	197·0	0·09	0·09	0·036	Nil
11	—	196·5	—	0·09	—	Nil

It will be apparent that this method of purification afforded a series of products displaying closely similar properties; indeed, bearing in mind the nature of glycogen and the difficulties attendant on the determination of its constants, we feel justified in regarding our purified specimens as consisting of a chemical individual. The small residual ash of the alkali-extracted specimens appeared to consist chiefly of silica, presumably introduced during the treatment with strong alkali in a glass vessel.

The effect of ions on the specific rotation.

Neither H nor OH ions, nor the presence of boric acid or borate, were found to affect the rotation of any of our specimens.

The presence of iron in glycogen.

After wet-ashing with pure nitric acid minute traces of iron were detected in most of the purified specimens. In two instances, specimens 8 and 9, the thiocyanate test, which was employed throughout, gave negative results even before the materials had been purified by acetic acid. From this evidence we conclude that iron does not form an essential part of the glycogen complex.

Aqueous acid hydrolysis of glycogen precipitated by acetic acid.

Carrying out polarimetric observations as before, we found that, whether water- or alkali-extracted material was under investigation, in every case the rotation-time curves were identical and uninflected, of the type of Fig. 1. This suggested that the inflections observed in the specimens which had been, at one time or another, treated with strong alkali, originated from the action of the latter substance on the ash soluble in acetic acid. To investigate this view, specimens purified by precipitation with acetic acid were subjected to the action of 30 % KOH as described earlier. On hydrolysis of these materials by aqueous acid no inflection could be detected in the rotation-time curves, thus confirming the above suggestion.

The evidence thus afforded shows that, when free from acid-soluble ash, the rotation-time curves of glycogen obtained by both methods of extraction are identical. We are thus finally able to state that, in so far as we are able to determine, strong alkali does not produce any chemical alteration in the carbohydrate.

GLYCOGEN FROM FASTED RABBITS.

The material was prepared by the aqueous extraction method and was obtained in a yield of 1·83 % of the initial liver weight. In Table IV details are given of the usual constants determined before and after precipitation from acetic acid solution.

Table IV.

	Before reprecipitation	After reprecipitation
$[\alpha]_D^{17°}$	+189·0°	+196·0°
Reducing power	0·26	0·19
Ash content %	0·66	0·13
Iodine coloration	Normal	Normal

On hydrolysis by aqueous acid the material yielded a smooth curve of the type of Fig. 1. These results are thus in no way significantly different from those obtained with the glycogen from fed animals.

RAT-LIVER GLYCOGEN.

The livers of fed rats were extracted with water by the method described in the section on rabbit-liver glycogen. The crude material was not examined but was purified by three precipitations from acetic acid (Table V).

Table V.

$[\alpha]_D^{17°}$	+196·0°
Reducing power	0·08
Ash content %	0·067

Iodine coloration: identical with that given by rabbit-liver glycogen

This specimen of glycogen was indistinguishable from the polysaccharide obtained from rabbit-liver, with the exception of a possible slightly greater opacity in aqueous solution.

Fish-liver glycogen.

Our material was obtained from fish of the genus *Gadus*, *G. morrhua* (cod), *G. aeglefinus* (haddock) and *G. merlangus* (whiting). The authors are indebted to Mr James Sharp, of the Torry Research Station of the Dept. of Scientific and Industrial Research, for a generous supply of crude material prepared from livers freshly removed from live fish in the following manner. The livers were digested with water at 70° and acetic acid added till the p_H was 4·5. After standing for some time the aqueous layer was run off from the supernatant oil and concentrated to small bulk, and the glycogen was precipitated by the addition of two volumes of 96 % alcohol.

The crude material, as we obtained it, contained much oil and protein. It was therefore exhausted first with boiling spirit, then with carbon tetrachloride. The residue was extracted four times with boiling water, the extract cooled and saturated with picric acid [Pertree and Alsberg, 1929]. The precipitated protein was centrifuged off and the yellow opalescent solution made up to 70 % alcohol by the addition of spirit. The precipitate was collected on a filter and washed with spirit till free from picric acid. It was then dissolved in water and treated with trichloroacetic acid as described above for rabbit-liver glycogen.

Carrying out experiments under conditions parallel with those on the material from rabbit liver we obtained the results given in Table VI.

Table VI.

	Initial	After KOH	After acetic acid
$[\alpha]_D^{17°}$	+196·0°	+194·2°	+196·6°
Reducing power	0·35	0·14	0·16
Ash content %	0·127	0·147	0·028

On hydrolysis by aqueous acid the material behaved exactly as the water-extracted material from rabbit-liver. The reducing power appeared to be slightly higher but we cannot yet say whether this is significant. The coloration produced by iodine was, however, distinctly different, being a deep yellow-brown.

Examination of the products of hydrolysis by aqueous acid.

In order to determine the nature of the units composing the complex of fish-liver glycogen, we decided to employ the process of direct hydrolysis of the polysaccharide by aqueous acid, rather than the now customary procedure of treating the acetate with methyl alcoholic hydrogen chloride and examination of the resulting glucosides [*cf.* Irvine and Hirst, 1924]. The reason for this choice lay in the fact that the yield of acetate obtainable from our material under the mildest conditions [Haworth and Percival, 1932] did not exceed 90 %, thus leaving a not inconsiderable portion of the material unaccounted for. As will be seen our final yield of hydrolysis product consisted of pure glucose amounting to 93·6 % of the starting material.

7·517 g. of purified glycogen were subjected to hydrolysis by 2·5 % aqueous hydrochloric acid. The rotation reached a constant value of +61·3° for the Hg green line after 5 hours. The acid was neutralised with silver carbonate, the solid removed by filtration and colloidal silver eliminated from the filtrate by treatment with hydrogen sulphide and norite. On evaporating the clear solution to

dryness *in vacuo* and drying to constant weight over P_2O_5, 7·818 g. of a light yellow glass were obtained, corresponding with a yield of hexose of 93·6 % of theory.

The crude material was dissolved in spirit and concentrated to a thick syrup under reduced pressure at 50°; the material then crystallised spontaneously. The whole was taken to dryness and recrystallised from 92 % alcohol. Several crops of crystals, amounting to nearly 92 % of the crude material, were collected, all showing identical microscopic appearance (small prisms), M.P. 144–145°. Mixed melting-points with authentic α-glucose (M.P. 144–146°) showed no depression. The united crystals showed in water the typical mutarotation curve of α-glucose.

The mother-liquors were taken to dryness under reduced pressure; the residue again crystallised spontaneously, as before, in small prisms, contaminated by a slight trace of a yellowish syrup. The M.P. was 127°. The rotation in water was considerably lower than that of the pure crystals, but the course of the downward mutarotation was identical with that of glucose. This portion of the investigation is detailed in Table VII.

Table VII.

Material	M.P.	Specific rotation: Initial (extrapolated)	Specific rotation: Final
α-Glucose	144–146°	+110°	+52·70°
Pure crystals (92 %)	144–146°	+110°	+52·59°
Residual material (8 %)	127°	+ 77°	+52·78°

There is thus no evidence of any units other than anhydroglucose being concerned in the structure of glycogen from this source.

Discussion.

From the results of our experiments on the action of strong KOH on highly purified glycogen, prepared in the absence of alkali, we are of the opinion that the carbohydrate is not chemically affected by the treatment. Thus, the suggestion of Haworth and Percival [1932] that alkaline treatment might oxidise a proportion of the terminal reducing groups present in the glycogen chains is in no way substantiated. Indeed, it appears to us that if the suggestions of these workers were correct one would expect to find gluconic acid derivatives among the fission products of their trimethylglycogen.

The observations of previous workers have shown considerable variation in the properties of their preparations of glycogen. We believe that our method of purification, involving precipitation by acetic acid, affords specimens displaying properties as constant as might be expected to be shown by a substance of the physical nature of glycogen. The specific rotations agree closely with one another, the ash contents are low, or zero, when precautions are taken to exclude contamination by silica *etc.*, and, in those specimens examined, the total phosphorus contents were found to be of a low order. The results obtained by previous observers are compared with those of the present authors in Table VIII.

The polarimetric behaviour of our materials on hydrolysis by aqueous acid shows that this process proceeds evenly throughout, there being no evidence of "breaks," which might be expected to occur if the reaction proceeded in different stages. The very closely agreeing figures which we obtained for these observations tend to support the view that our specimens consisted of the same individual

Table VIII. *Liver glycogens.*

Authors	Source	$[\alpha]_D$	Method of purification	% Ash	% P_2O_5
Gatin-Gruzewska [1904]	Dog	+197°	16–18 pptns. from alkali	Nil	—
Harden and Young [1902]	Rabbit	+191°	—	—	—
Norris [1913]	Rabbit	—	Dialysis	0·51	—
,,	Dog	—	,,	0·38	—
Sayhun and Alsberg [1930]	Rabbit	—	Precipitation	0·20	0·032
Haworth *et al.* [1929]	?? (Kahlbaum)	+191°	Electrodialysis	0·37	0·14
Present authors	Rabbit (fed)	+196°	Pptn. by acetic acid	Nil	0·107
,, ,,	Rabbit (fasted)	+196°	,, ,,	0·130	—
,, ,,	Rat	+196°	,, ,,	0·067	—
,, ,,	Fish	+196°	,, ,,	0·028	—

substance. The dissimilarity in iodine colorations developed by mammalian and fish glycogens, although distinct, may not be associated with profound differences in chemical structure.

Further work in this field is at present in progress.

Summary.

1. Criteria for liver glycogen have been determined, *viz.* specific rotation, reducing power, ash content, coloration with iodine and polarimetric behaviour on hydrolysis by aqueous acid.

2. Judged by these criteria, the liver glycogens from the following sources are not significantly different: fasted rabbits, fed rabbits, fed rats and fish of the genus *Gadus*.

3. Glycogen may be obtained in a highly purified, ash-free state by precipitation from water by acetic acid.

4. The chemical properties of highly purified glycogen are not altered by treatment with 30 % KOH at 100°.

The authors desire to express their gratitude to Prof. J. J. R. Macleod for his interest and for the facilities which he provided for this work, and to Sir J. C. Irvine for his generous advice and criticism. Part of the expense of this investigation was defrayed by a grant from the Government Grants Committee of the Royal Society to one of us (F. G. Y.), for which we wish to record our thanks.

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CXXVI. AN ELECTRIC FURNACE FOR MICRO-KJELDAHL DIGESTIONS AND SIMILAR PURPOSES.

By SYDNEY JOHN FOLLEY.

From the National Institute for Research in Dairying, University of Reading.

(*Received March 24th, 1934.*)

In carrying out micro-analyses on blood involving Kjeldahl and other incinerations the need for a suitable furnace was felt, and the apparatus described and illustrated in this note was therefore devised. Electrical heating was preferred to gas on account of its convenience and for another reason mentioned below.

The frame is of angle iron screwed to a teak base. The flasks are supported at an angle of 45° on a perforated sheet of asbestos, their ends projecting for about $\frac{1}{4}$ in. into openings cut in the lead pipe, *A*. A platform made from sheet asbestos supported by strips of angle iron carries two Ferranti 500 watt heating coils, which are held in place by the spring clips, *C*. Each coil serves to heat four flasks. The copper reflectors, *D*, shield the heating elements from draughts and greatly increase the available heat. The platform carrying the heaters is pivoted at the back, and the position of the heating coils relative to the flasks can be altered by raising or lowering the wing nuts, *F*. This arrangement affords a means of adjusting the rate of heating.

The fume absorber, *A*, which slopes slightly from left to right (Fig. 1) is fixed to uprights of spring steel so that its position relative to the flasks can be adjusted by means of the wing nuts, *H*.

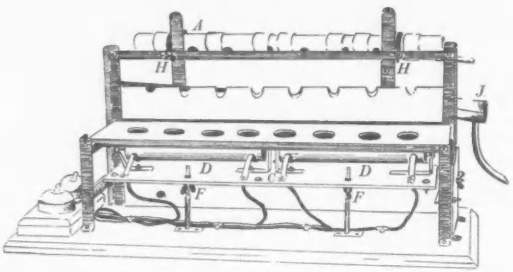

Fig. 1.

The furnace can be safely used in a laboratory without a fume cupboard since when the lead pipe is connected to a good water pump and the unoccupied holes closed by lead slides no fumes escape into the atmosphere.

If the digestion mixture contains an appreciable quantity of water, Kjeldahl flasks are often cracked by water which condenses in the lead pipe and runs down the outside of the flasks. This can be prevented by placing rubber washers

round the flasks at a suitable height so that the condensed liquid drips into the metal trough, *J*, which should be coated with an acid-resisting paint.

In the author's experience micro-Kjeldahl digestions proceed much more smoothly with an electrically heated than with a gas-heated furnace. This may be due to more even heating of the contents of the flasks in the former case. Using the apparatus described for the digestion of whole blood it has been found that when the heating is suitably regulated no bumping takes place. Even with whole milk, a notorious offender in this respect, the amount of bumping is quite insignificant. Moreover, a sample of powdered dried grass, kindly submitted by Dr A. W. Greenhill of the Agricultural Research Station, Jealott's Hill, Berks, in the digestion of which over gas flames excessive bumping had been experienced, was digested on this apparatus with no bumping whatever.

SUMMARY.

An electric furnace suitable for micro-Kjeldahl and similar incinerations is described. The use of an electric furnace provided with a means of regulating the heating eliminates bumping.

I desire to thank N. Gruber for preparing the drawing and P. W. Batten for his assistance in building the furnace.

The apparatus can be obtained from Messrs Callas Sons and May Ltd., 65 Oxford St, Reading.

CXXVII. THE OXIDATION OF THE FATTY DIBASIC ACIDS AND OF LAEVULIC ACID BY HYDROGEN DIOXIDE IN PRESENCE OF A CUPRIC SALT.

By ANNIE PHYLLIS PONSFORD and IDA SMEDLEY-MACLEAN.

From the Biochemical Department, Lister Institute, London.

(*Received May 1st, 1934.*)

Considerable quantities of the dibasic acids are formed in the laboratory when the higher fatty acids are oxidised by such reagents as alkaline permanganate or nitric acid; and oxalic, succinic, adipic and suberic acids have been identified. It is however generally accepted that *in vivo* the path of the oxidation of the fatty acids does not proceed by way of the dibasic acids. Verkade and Van der Lee [1934] have however recently shown that when decanoic or undecanoic acid was taken by mouth, the terminal methyl group was oxidised and the corresponding dibasic acid excreted in the urine. In the laboratory, oxidation of the higher fatty acids with hydrogen dioxide is the process which has been supposed most closely to resemble the normal process of oxidation in the body, and recent experiments [Smedley-MacLean and Pearce, 1934] on the oxidation of palmitic acid, in which this process was catalysed by the addition of a cupric salt, have shown that at 90° there is a rapid breakdown, the end-products being succinic, carbonic and the volatile fatty acids. We therefore investigated the oxidation of the dibasic acids with the same reagents. These acids were found to be more readily attacked than the monobasic acids, so that even at 60° no glutaric or adipic acid and only 10 % suberic acid remained unchanged after 1 hour's heating. When palmitic acid was oxidised by excess of hydrogen dioxide in the presence of copper, there was very little evidence of keto-oxidation: the main products were hydroxy-acids, and there was also evidence of a considerable amount of desaturation. With the dibasic acids, definite evidence was obtained of keto-oxidation: thus laevulic acid was formed from adipic acid, a small quantity of acetonylacetone from suberic acid, and acetone was isolated from the products of oxidation of glutaric acid. With suberic acid however there was definite evidence of hydroxylation, the α-hydroxy-derivative and a lactonic acid being isolated.

From all the acids investigated (laevulic, suberic, adipic and glutaric), succinic acid was isolated as an oxidation product, and as succinic acid is itself oxidised under the conditions of the experiment giving a considerable proportion of carbonic and other volatile acids, the succinic acid isolated in these oxidation experiments must represent only a proportion of that actually formed. No evidence of the formation of fumaric, malic or tartaric acid was obtained.

The method of oxidation was that described by Smedley-MacLean and Pearce [1934], the volatile products being separated by steam-distillation.

The proportion of dibasic acids oxidised by a quantity of hydrogen dioxide sufficient to give about 19 % more than the amount of oxygen necessary for the complete oxidation of the acid was considerably augmented by the addition of

Table 1. *Oxidation of certain dibasic acids and laevulic acid carried out for 1 hour at 60°.**

	Conditions				In absence of copper					In presence of copper						
Acid oxidised	Weight of acid g.	Total vol. ml.	Acidity (normality)	No. ml. 6 % H_2O_2	CO_2 % exp.†	CO_2 % distillation‡	Reducing volatile acid calc. as % formic	Remaining volatile acids calc. as % acetic	Ether extract g.	CO_2 % exp.†	CO_2 % distillation‡	Reducing volatile acid calc. as % formic	Remaining volatile acids calc. as % acetic	Ether extract g.	Acetone g.	Aldehyde calc. as acetic g.
Succinic	2·5	300	0·0142	96	3·0	1·9	1·6	4·6	2·2	10·6	2·0	2·6	6·8	2·2	—	—
	2·5	300	0·0142	96	1·3	2·1	0·9	22·5	1·7	9·5	4·0	5·0	18·4	1·3	—	—
	2·5	300	0·0140	204	—	—	—	—	—	29·4	13·9	8·1	13·8	>0·3	—	—
	2·5	515	0·0242	350	—	—	—	—	—	45·6	10·1	1·8	14·0	0·6	—	—
Glutaric	2·5	300	0·0142	122	0·6	0·5	0·1	3·5	2·1	26·2	4·8	4·7	16·7	>1·4	—	—
	2·5	515	0·0346	350	—	—	—	—	—	53·3	13·1	2·8	40·2	0·3	—	—
	2·5	515	0·0242	350	—	—	—	—	—	42·7	27·9	6·2	48·0	1·0	0·004	0·059
											§35·9		§22·0			
	2·5	515	0·0242	350	—	—	—	—	—	44·1	14·3	5·9	46·3	1·0	0·042	0·031
											§20·4		§25·4			
Adipic	2·5	300	0·0041	150	2·0	7·1	0·04	5·2	2·4	4·0	3·8	2·0	8·3	2·2	—	—
	2·5	300	0·0142	144	0·5	6·8	0·2	3·1	2·2	15·0	7·3	5·4	8·6	1·8	—	—
	2·5	515	0·0222	350	0·2	2·7	0·2	4·1	2·1	32·2	17·0	4·0	24·2	0·6	—	—
	2·5	515	0·0222	350	0·1	5·2	0·4	11·4	—	30·9	14·6	4·4	24·4	0·7	—	—
	2·5	515	0·0222	350	—	—	—	—	—	22·2	13·7	4·0	21·8	0·4	—	—
Suberic	2·5	515	0·0186	350	0·2	1·2	0·1	1·2	2·1	5·3	6·2	4·8	7·1	1·4	—	—
	2·5	515	0·0186	350	—	—	—	—	—	7·8	9·5	4·2	10·3	1·9	—	—
	2·5	515	0·0275	350	—	—	—	—	—	9·8	8·1	7·6	17·7	1·6	0·010	0·008
											§8·8		§15·2			
	2·5	515	0·0242	350	—	—	—	—	—	8·7	7·1	3·7	22·1	1·5	0·015	0·020
Azelaic	2·5	515	0·0242	350	—	—	—	—	—	8·8	4·3	6·3	41·4	1·8	0·017	0·010
											§9·0		§24·3			
Laevulic	2·5	515	0·0142	350	—	—	—	—	—	14·3	17·9	2·9	34·8	1·2	—	—
	2	515	0·0058	350	—	—	—	—	—	15·7	11·9	2·2	29·1	0·8	—	—

* Percentages given are % carbon of original acid converted into the product concerned.
† Carbon dioxide evolved during the 1 hour's oxidation.
‡ Carbon dioxide evolved during subsequent steam-distillation after destruction of H_2O_2.
§ The lower figures give corrected values, allowance having been made for CO_2 retained as carbonate in the neutralised volatile acid distillates.

a cupric salt to the mixture; at the same time the proportion converted into carbonic and the lower fatty acids was much increased, the effect being most striking in the case of glutaric acid.

It will be seen from the figures given in Table I for adipic acid that a large increase in the amount of the hydrogen dioxide in the reaction mixture produced very little further oxidation in the absence of the copper salt. This agrees with the observation made by Dakin [1908] on the oxidation of the monobasic saturated fatty acids. Taking the percentage of the original acid converted into CO_2 and volatile acids as the criterion of the extent of oxidation, in the presence of the cupric salt, glutaric and adipic acids were more readily oxidised than succinic acid, both with a slight and with a large excess of the dioxide: in the latter case glutaric acid was almost completely converted into carbonic and the lower volatile fatty acids. A certain amount of carbonic acid was always given off during the steam-distillation of the product, markedly so in the case of glutaric and laevulic acids, probably owing to the decomposition of some unstable intermediate substance. Very little formic acid was present. Acetone in small amount was detected in the neutral volatile distillate after oxidation of glutaric acid; since acetone is hardly attacked by hydrogen dioxide in the presence of a copper salt, it is probable that little of the glutaric acid had undergone β-keto-oxidation.

When adipic acid was treated with a large excess of the dioxide in the presence of copper, no unchanged adipic acid was detected in the ether extract, but from 10 to 13 % of the total carbon had been converted into succinic and laevulic acids in the proportion of roughly 9 : 1. The succinic acid was identified by a mixed melting-point determination and the laevulic acid by the melting-point of its dinitrophenylhydrazone (200–202°), and a mixed melting-point with that of the same compound prepared from pure laevulic acid and melting at 202–204°. The formation of laevulic from adipic acid would be explained by oxidation at either the β- or γ-carbon atom, since in the dibasic adipic acid the β- and γ-derivatives are identical.

From the oxidation product of laevulic acid itself, carried out under similar conditions, small quantities of succinic acid and of acetone were obtained. Since on oxidation under these conditions, laevulic gave considerably less succinic acid than did adipic acid, laevulic acid was probably not the only intermediate substance formed. Succinic acid would also be formed by oxidation of the two α-carbon atoms, or possibly of the α- and δ-atoms, the α- and δ-positions in adipic acid being like the β- and γ-positions interchangeable. In the formation of succinic from glutaric acid, oxidation must have occurred at the α-atom (identical with the δ-position). The isolation of succinic acid from the higher dibasic acids in very much the same proportion is possibly due to a process of γ-oxidation by which a molecule of this acid is separated.

With a large excess of the dioxide, in the presence of a cupric salt at 60°, suberic and azelaic acids were less completely oxidised than the dibasic acids of lower molecular weight.

Under the above conditions from 20 to 40 % of suberic acid was converted into carbonic and the lower volatile fatty acids. The first two litres of the steam-distillate were neutralised and again distilled, the neutral distillate being collected as long as it gave a precipitate with dinitrophenylhydrazine hydrochloride solution. The first 200 ml. of this distillate were tested for acetaldehyde, and the subsequent distillate when treated with the reagent gave a hydrazone melting at 232–234°: this was very insoluble in most organic solvents but could be dissolved in glacial acetic acid and precipitated from this by water.

Microanalysis: (Found: C, 45·58; H, 3·72; N, 22·90 %. $C_{18}H_{18}N_8O_8$ requires C, 45·57; H, 3·80; N, 23·62 %). It was therefore identified as the di-dinitrophenylhydrazone of acetonylacetone: this substance was then prepared and the identity of the products established by determination of a mixed melting-point.

$\beta\beta$-Oxidation must therefore have taken place and acetonylacetone produced by elimination of CO_2 from the diketosuberic acid formed.

$$COOH.CH_2.CH_2.CH_2.CH_2.CH_2.COOH \rightarrow COOH.CH_2.CO.CH_2.CH_2.CO.CH_2.COOH \rightarrow CH_3.CO.CH_2.CH_2.CO.CH_3$$

From the glacial acetic mother-liquors, a very small amount of a more soluble hydrazone, melting unsharply at 100°, was obtained, but the quantity was too small for further investigation. In the preparation of the dinitrophenylhydrazone from acetonylacetone, after the separation of the di-dinitrophenylhydrazone melting at 245°, a second hydrazone melting at 167° was separated from the glacial acetic mother-liquors.

The products from four experiments in each of which 5 g. of suberic acid had been oxidised were worked up together and from these a total of 12·06 g. of an ether-soluble non-volatile acid product were obtained. This was neutralised with *N* NaOH, the equivalent of the acid being 86.

No residue was obtained from the ethereal extract of the neutral solution. After acidification with hydrochloric acid, the bulk of the acids were removed by ether extraction, the aqueous solution evaporated to dryness and the residue extracted first with ether and then with absolute alcohol.

The material soluble in ether was washed three times with chloroform, leaving 4·06 g. of an insoluble crystalline acid, giving both on titration and on analysis of the silver salt an equivalent of 71. By fractional crystallisation from water of similar material, two substances were separated: (1) less soluble crystals identified by a mixed melting-point with suberic acid, and (2) a readily soluble substance melting at 178° which did not depress the melting-point of pure succinic acid when added to it. The 4·06 g. of material insoluble in chloroform, which had an equivalent of 71, probably therefore consisted of approximately 2·38 g. succinic and 1·68 g. unchanged suberic acid. About 10 % of the original suberic acid had remained unoxidised.

The chloroform solution contained 4·84 g. of a yellow oil, of which 2·35 g. were soluble in benzene: the oily residue from the benzene solution on standing in an evacuated desiccator deposited colourless crystals of which 0·13 g., M.P. 109–111°, remained after draining on a porous tile. The equivalent of these determined by titration was 84 and by analysis of the silver salt 93. Similar crystals were obtained in another experiment but with an equivalent of 102 (calculated from the decomposition of the silver salt).

Analysis: (Found: C, 50·63; H, 6·60 %. $C_8H_{14}O_5$ requires C, 50·52; H, 7·37 %. Equivalent, 95. $C_8H_{12}O_5$ requires C, 51·06; H, 6·33 %. Equivalent, 94).

The solution of the sodium salt gave no precipitate with dinitrophenylhydrazine hydrochloride and did not decolorise potassium permanganate solution; the melting-point of a specimen of α-hydroxysuberic acid (M.P. 110–112°) did not appear to be appreciably depressed by the addition of a small amount of the crystals now isolated, so that in spite of the low hydrogen percentage these crystals may have consisted of α-hydroxysuberic acid.

The tile which had been used for draining these crystals was extracted with ether, and an oil sparingly soluble in light petroleum was obtained. Its equivalent determined by titration in the cold was 116: by warming with excess of *N*/2

NaOH and titrating the excess with $N/2$ H_2SO_4 the equivalent was found to be 85. Decomposition of the moderately soluble silver salt gave an equivalent of 86. The high equivalent obtained by titration in the cold suggested the presence of a certain amount of the lactone of a hydroxydibasic acid.

	% C	% H	Equivalent
Microanalysis: Found	49·57	6·96	86
Hydroxysuberic acid ($C_8H_{14}O_5$)	50·52	7·37	95
Lactone of hydroxysuberic acid	55·82	6·98	172
Hydroxypimelic acid ($C_7H_{12}O_5$)	47·73	6·82	88
Lactone of hydroxypimelic acid	53·16	6·38	158

A mixture of 2/3 hydroxypimelic and 1/3 of the corresponding lactone contains 49·54 % C, 6·67 % H and would have an equivalent of 111, which would diminish to 88 on opening the lactone ring. These figures agree fairly well with those actually found but we were unable to find any evidence of pimelic acid in the reduction product. Possibly the low carbon percentage was due to the admixture of succinic acid or other impurity with hydroxysuberic acid.

0·227 g. of the oil was boiled for 3 hours with 5 ml. acetic anhydride, 2·5 g. hydriodic acid (sp. gr. 1·7) and 0·25 g. red phosphorus. The mixture was cooled and diluted: a tarry oil separated and the whole was evaporated nearly to dryness under reduced pressure. The black residue was dissolved in alcohol, excess of phosphorus filtered off and the dark brown filtrate boiled for 1½ hours with 6 g. zinc dust and 10 g. glacial acetic acid. The filtered solution was concentrated to about 10 ml. and then considerably diluted, causing the separation of an oil, which was extracted by ether and saponified by boiling with 40 ml. 5 % alcoholic potash for 1 hour. After diluting the product with water, ether extraction yielded about 0·06 g. of a white crystalline neutral substance melting at 57–58° when recrystallised from benzene and alcohol. This was slightly soluble in water the solution giving traces of a precipitate with a solution of dinitrophenylhydrazine hydrochloride and a slight silver mirror with ammoniacal silver nitrate.

Microanalysis: (Found: C, 76·57; H, 12·86 %. $C_{10}H_{20}O$ requires C, 76·93; H, 12·82 %. $C_9H_{18}O$ requires C, 76·05; H, 12·68 %). No further evidence as to its constitution was obtained.

After acidifying the alkaline aqueous solution, ether extraction yielded about 0·12 g. of a white crystalline acid which after recrystallisation melted at 106–108° and dissolved in water to the extent of less than 1 %, thus resembling in its properties the α-hydroxysuberic acid, crystals of which had originally been separated from this oily product: pimelic acid melts at 103° and is about 5 % soluble in water; the melting-point of suberic acid is 139–140° and its solubility less than 1 %.

On the whole the evidence points to hydroxylation of the suberic acid having taken place in the α- and γ-positions.

From the chloroform solution 2·04 g. of an oil insoluble in benzene were obtained which after standing for some time in an evacuated desiccator deposited 0·64 g. colourless crystals. These were separated on a porous tile and recrystallised from benzene: they melted at 130–131° and their equivalent determined by titration in the cold was 102, by analysis of the white silver salt, which was comparatively stable to light, 99. A solution of the sodium salt gave no hydrazone when treated with a solution of dinitrophenylhydrazine hydrochloride and did not decolorise a solution of potassium permanganate, so that the presence of a saturated hydroxy-acid was inferred.

Microanalysis of the silver salt: (Found: Ag, 52·11; C, 24·22; H, 2·70 %. $C_8H_{12}O_5Ag_2$ requires Ag, 53·46; C, 23·76; H, 2·97 %).

The percentage of silver was estimated from the weight of the residue in the boat after combustion. The melting-point was considerably higher than that of α-hydroxysuberic acid and since there was no evidence of lactone formation, the presence of γ-hydroxysuberic acid could be excluded. β-Hydroxysuberic acid, not hitherto described, may therefore have been present.

The oil from which these crystals had been separated contained 44·54 % C and 6·66 % H: the equivalent by titration was 98, by analysis of the silver salt 94: $C_7H_{12}O_6$ requires 43·75 % C; 6·25 % H; equivalent, 103. A dihydroxypimelic acid may therefore have formed the bulk of the oil.

On adding a solution of its sodium salt to a solution of dinitrophenylhydrazine hydrochloride, a very small amount of a precipitate, M.P. 85–90°, formed.

Microanalysis: (Found: C, 45·66; H, 4·01; N, 16·01 %. $C_{14}H_{16}O_8N_4$ (the dinitrophenylhydrazone of ketosuberic acid) requires C, 45·65; H, 4·35; N, 15·22 %); traces of a ketosuberic acid were therefore present in the oil.

The residue of the original oxidation product, which was insoluble in ether, was extracted with alcohol: most of this extract was soluble in water and consisted of a gummy substance which only very slowly reached constant weight and the nature of which was not determined.

The small amount of water-insoluble residue dissolved readily in alcohol and yielded a low-melting dark brown substance the constitution of which was not determined.

SUMMARY.

1. Laevulic acid and the dibasic acids, succinic, glutaric, adipic, suberic and azelaic, were readily oxidised at 60° by hydrogen dioxide in the presence of a cupric salt, a considerable proportion being broken down to carbonic and the lower volatile fatty acids.

2. Succinic acid was identified as a product of oxidation of glutaric, adipic, suberic and laevulic acids.

3. Azelaic and suberic acids were less completely broken down than the lower acids.

4. From glutaric acid, a small amount of acetone was isolated, showing that β-keto-oxidation had taken place.

5. From both glutaric and laevulic acids, considerable amounts of CO_2 were obtained during the steam-distillation of the oxidation product, pointing to the presence of an unstable intermediate product.

6. From adipic acid, laevulic acid was obtained in appreciable amount, showing that β-keto-oxidation had taken place.

7. Only in the case of suberic acid was definite evidence forthcoming of the presence of hydroxy-derivatives. The evidence available pointed to the presence of α- and γ-hydroxysuberic acids and possibly of the β-hydroxy-derivative. The presence of hydroxy-derivatives of pimelic acid as intermediate products could not be excluded. The identification of a very small amount of acetonylacetone showed that some ββ-keto-oxidation had taken place. The occurrence of a monoketosuberic acid and possibly of other keto-derivatives was also indicated.

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CXXVIII. STUDIES ON REVERSIBLE DEHYDROGENASE SYSTEMS.

I. THE REVERSIBILITY OF THE HYDROGENASE SYSTEM OF *BACT. COLI*[1].

By DAVID EZRA GREEN and LEONARD HUBERT STICKLAND[2].

From the Biochemical Laboratory, Cambridge.

(*Received May 1st, 1934.*)

Stephenson and Stickland [1931] described an enzyme in various bacteria which could catalyse the reduction of oxygen, methylene blue, nitrate, sulphate or fumarate by molecular hydrogen. Consideration of this catalysis discloses a striking resemblance between hydrogenase and platinum black. Both the enzyme and the heavy metal catalyse the reaction

$$H_2 \rightleftharpoons 2H \rightleftharpoons 2H^{\cdot} + 2\epsilon.$$

In the presence of platinum black, this reaction proceeds reversibly (*cf.* the use of platinised platinum electrodes for the measurement of p_H). The experiments which follow show that the same equilibrium point between molecular and ionic hydrogen is reached in the presence of *Bact. coli* as in the presence of platinum black.

Experimental methods.

Bact. coli was grown in Roux bottles on a broth agar in the usual fashion. After two washings by centrifuging, the thick mass of bacteria was treated with saturated toluene water at room temperature for 15 minutes. This treatment sufficed to eliminate completely all endogenous reducing power.

The equilibrium between H_2 and $H^{\cdot}$ was measured with methylviologen (dimethyl-$\gamma\gamma'$-dipyridylium chloride), an indicator which lies in the range of the hydrogen electrode between p_H 7 and 9. The indicator was prepared according to the directions of Michaelis and Hill [1933], and the recrystallised chloride gave theoretical figures on elementary analysis.

The equilibrium potential was measured both colorimetrically and electrometrically. The colorimetric technique consisted essentially in setting up Thunberg tubes each containing buffer, bacterial suspension and methylviologen. The tubes were evacuated thoroughly and filled with oxygen-free hydrogen at a definite partial pressure according to the method of Stephenson and Stickland [1931]. Since the reaction proceeds very rapidly at 40°, the tubes were incubated at that temperature for an hour with frequent vigorous shaking. Then the temperature was lowered to 30° for the final measurements, at least 2 hours being allowed for equilibrium to be reached. For estimation of the degree of reduction a set of standards was prepared containing various fractions of the

[1] A preliminary note has already appeared in *Nature* (1934), **133**, 573.

[2] Beit Memorial Research Fellow.

total amount of indicator solution used in the experimental tubes. Both the total volume of fluid and the volume of bacterial suspension were the same in all the standard and experimental tubes. Reduction of the indicator in the standards was effected by adding a few crystals of sodium hydrosulphite and an excess of solid sodium carbonate.

The electrode vessels for the electrometric measurement of the equilibrium were constructed according to the design of Borsook and Schott [1931]. The only technical difficulty was the preparation of an agar bridge which could withstand a vacuum. By elimination of undissolved particles and air bubbles from the molten agar, bridges could be made to withstand a pressure of one atmosphere for the duration of several days, but the reliability rarely extended beyond the first few experiments.

The setting up of the experiment in the electrode vessel was identical with that described for the colorimetric experiments. Six electrode tubes were mounted on a special rack in an air-thermostat maintained at 30°. This rack permitted of vigorous shaking of the tubes. All the six tubes dipped into a large beaker of saturated potassium chloride which made contact with a reference saturated calomel electrode.

The potentiometry is described in detail in a paper by Green [1933]. The presence of the bacteria renders the potential recorded at the electrode somewhat unsteady, and readings of the potentiometer can at best be correct to about 1 millivolt.

The p_H of the buffer solutions used was measured by the quinhydrone and hydrogen electrodes. The buffers were made up to a concentration of $M/5$, and the final p_H of the bacterial suspension was assumed to be identical with that of the buffer alone.

Experimental results.

If *Bact. coli* and colloidal palladium are equivalent as catalysts, then for the same p_H and partial pressure of hydrogen the extent of reduction of methylviologen should be the same whichever catalyst is used. Table I summarises the results of this comparison. One set of Thunberg tubes contained 2 ml. of $M/5$ buffer, 0·5 ml. of $M/1000$ methylviologen and 1 ml. of bacterial suspension. A duplicate set was prepared in the same way except that the bacterial suspension had been previously heated to 100° for 15 minutes to inactivate the hydrogenase completely, and a drop of colloidal palladium suspension had been added. It is

Table I.

		Degree of reduction (%)	
p_H	Pressure of H_2 (mm. of Hg)	*Bact. coli* as catalyst	Palladium as catalyst
7·32	498	29	28
7·32	200	24	22
7·83	199	37	37
7·83	81	26	27
8·33	80	51	51
8·33	29	37	40
8·96	30	56	56
8·96	10·5	45	45

clear that the equilibrium-point is identical in the two sets of tubes. These measurements represent the average of comparisons carried out by two independent observers, and with the exception of one reading the two sets of values agreed to within 5 %.

If the potentials corresponding to the observed degree of reduction in Table I are calculated from the E_0 of the indicator, a uniform discrepancy of about 20 millivolts is found between the observed and theoretical potentials for the system $H_2 \rightleftharpoons 2H^{\cdot} + 2\epsilon$. This can only mean that the assumption of Michaelis and Hill [1933] that the E_0 of methylviologen (−0·446 v.) is independent of p_H does not hold below p_H 9. Actually the measurements of these authors were confined to fairly alkaline p_H values, and their value for the neutral range is simply an extrapolation. One of us (D. E. G.) has noticed a similar discrepancy between the colorimetric and potentiometric values for the xanthine oxidase system in presence of methylviologen.

Table II contains a summary of the potentiometric measurements of the equilibrium $H_2 \rightleftharpoons 2H^{\cdot} + 2\epsilon$ in presence of *Bact. coli* and methylviologen, with

Table II.

p_H	Pressure of H_2 (mm. of Hg)	Potential (volts) Observed	Theoretical	Difference
7·02	600	−0·401	−0·408	+0·007
7·71	100	−0·433	−0·439	+0·006
7·71	306	−0·448	−0·453	+0·005
7·71	428	−0·455	−0·458	+0·003
8·12	49	−0·452	−0·455	+0·003
8·12	109	−0·463	−0·464	+0·001
8·12	161	−0·470	−0·470	0·000
8·12	237	−0·477	−0·475	−0·002
8·12	598	−0·489	−0·487	−0·002
8·23	105	−0·464	−0·470	+0·006
8·23	138	−0·475	−0·473	−0·002
9·10	20	−0·484	−0·487	+0·003

variation of the hydrogen and hydrogen ion concentrations. The remarkable agreement between the observed values of the potential and those calculated from the hydrogen electrode equation

$$E_h = -\frac{RT}{F} p_H - \frac{RT}{2F} \log P_{H_2},$$

gives adequate proof that hydrogenase functions as a perfect catalyst, *i.e.* it increases the rate of attainment of the equilibrium but does not alter the equilibrium-point.

SUMMARY.

Bact. coli has been shown to catalyse the reaction

$$H_2 \rightleftharpoons 2H^{\cdot} + 2\epsilon$$

in a completely reversible way. The hydrogenase system is the most negative reversible oxidation-reduction system as yet described in living cells.

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CXXIX. THE ACTION OF INHIBITORS ON THE CATECHOL OXIDASE OF POTATOES.

By DEREK RICHTER.

From the Biochemical Laboratory, Cambridge.

(*Received April 11th, 1934.*)

Attempts have been made to classify the phenolases into groups such as the *catechol oxidases*, *tyrosinases*, and *laccases* [Raper, 1928], but the classification is made difficult by the lack of satisfactory methods of characterising the enzymes [Onslow and Robinson, 1928].

The present work was undertaken with the object of testing whether the catechol oxidases of different origin behave similarly towards inhibitors, or whether they show any marked differences of behaviour which might be used as a further guide in classifying these enzymes.

Catechol oxidases are found widely distributed in plants, fungi and insects. In the animal kingdom, a phenolase specific for 3 : 4-dihydroxyphenylalanine ("Dopa oxidase") and a phenolase of horse's heart muscle [Wieland and Lawson, 1931] have been investigated. Other systems effecting the oxidation of adrenaline and phenolases present in melaniferous tumours have been described. Summaries of the literature are given by Raper [1928], Onslow and Robinson [1928], Hizume [1924] and Oppenheimer and Kuhn [1931].

Resorcinol was chosen as a suitable inhibitor, since preliminary experiments with the catechol oxidase of potatoes showed that the inhibition with resorcinol appeared to be of a simple competitive type, as it could be reduced by increasing the substrate concentration.

Experimental.

Cathechol oxidase of potatoes. The catechol oxidase of potatoes (called tyrosinase by Raper [1928]) was prepared by the method of Szent-Györgyi [Keilin, 1928] and was further purified by adsorption on aluminium phosphate at p_H 6 and elution with phosphate buffer at p_H 8. The aluminium phosphate [Tsuchihashi, 1923] was added to the enzyme solution until most of the enzyme was adsorbed. (This was shown by centrifuging off a small sample of the clear solution and testing for catechol oxidase by shaking in a test-tube with a few drops of 2 % catechol.) After adsorption, the aluminium phosphate was centrifuged off and washed twice with distilled water, and the enzyme was eluted by adding disodium hydrogen phosphate solution until the mixture was at p_H 8. About 50 % of the enzyme was lost in the process of adsorption, but the resulting preparation, which was nearly colourless, appeared to be very pure, and could be kept for several weeks in the ice-chest without any considerable loss of activity.

This preparation brought about a very rapid oxidation of catechol and its derivatives, but the system was found to be unsuitable for making kinetic measurements as the reaction came to a standstill after the first few minutes owing to the inhibition of the system by one of the reaction products (Fig. 1).

This auto-inhibition was probably due to the *o*-quinone formed in the reaction

$$C_6H_4(OH)_2 + O_2 \rightarrow C_6H_4O_2 + H_2O_2$$

since it was greatly reduced by adding to the system substances such as aniline or *o*-phenylenediamine which readily combine with *o*-quinones. Other substances found to be effective in decreasing the auto-inhibition were *m*- and *p*-phenylenediamine, glycine and alanine (Fig. 1).

For the purpose of making kinetic measurements with inhibitors *M*/20 aniline was therefore added to the system, which in the presence of *M*/125 catechol, and *M*/50 phosphate buffer p_H 6·8, gave a regular and reproducible reaction velocity. The anilinoquinone formed during the reaction separated out as a red precipitate.

Enzyme strength. As an arbitrary unit of the enzyme strength the amount of enzyme was chosen which in the presence of *M*/125 catechol, *M*/50 phosphate buffer p_H 6·8, and *M*/20 aniline (total volume 5 ml.) gave an oxygen uptake of 100 μl. in 10 minutes when shaken in air in a Barcroft apparatus at 20°. This enzyme unit is referred to as Catechol Unit or C.U. throughout the text.

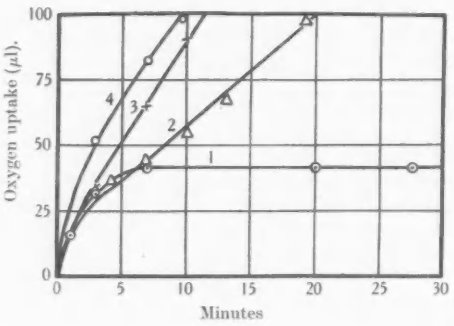

Fig. 1.

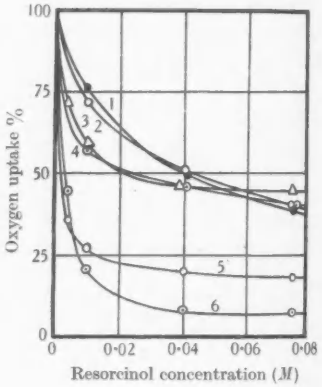

Fig. 2.

Fig. 1. In each experiment *M*/125 catechol, *M*/50 phosphate buffer p_H 6·8, and 0·9 C.U. potato catechol oxidase. Total volume 5 ml. Temperature 20°. 1. No addition. 2. *M*/10 glycine. 3. *M*/20 aniline. 4. 0·4 % *o*-phenylenediamine.

Fig. 2. In each experiment *M*/125 catechol, *M*/50 phosphate buffer p_H 6·8, *M*/20 aniline. Total volume 5 ml. Temperature 20°. 1. *Polyporus hispidus*. 2. *Tenebrio molitor*. 3. Potato. 4. *Agaricus campestris*. 5. *Sambucus nigra*. 6. *Syringa vulgaris*.

Inhibition by resorcinol.

The enzyme preparations used for comparison were prepared by the alcohol extraction method of Onslow and Robinson [1928] from fresh specimens of *Syringa vulgaris* (lilac), *Sambucus nigra* (elder), *Agaricus campestris* (mushroom), and *Polyporus hispidus*. The preparation from *Tenebrio molitor* (mealworm) was purified by repeated precipitation with alcohol and dialysis.

The enzymes were all used at approximately the same strength (0·3 C.U./ml.) under the conditions described for determining the enzyme strength of the

potato catechol oxidase, and the oxygen uptake in the presence of a series of concentrations of resorcinol was measured. The oxygen absorbed between the third and the thirteenth minutes was taken for comparison. The oxygen uptake plotted against the inhibitor concentration gave the curves shown in Fig. 2.

The inhibition curves obtained in this way appeared to depend very little on the degree of purity of the enzyme, since the curves obtained with crude potato juice agreed closely with the curves for the highly purified enzyme.

It can be seen at once from the curves (Fig. 2) that the catechol oxidases from different sources are not identical, but show marked specific differences in their behaviour. It is noticeable that the curves for *Syringa vulgaris* and *Sambucus nigra*, neither of which contains tyrosinase, lie together. While the usual methods of characterising enzymes, such as the measurement of p_H optimum or thermolability, must depend largely on the nature of the accompanying colloids, the characteristic selective inhibition of the phenolases by an inhibitor such as resorcinol probably depends more on the specific nature of the active centres of the enzymes.

Other inhibitors.

Apart from the inhibition by H_2S and HCN described by Keilin [1928] the catechol oxidase of potatoes is comparatively resistant towards narcotics and the usual enzyme poisons. The action of a number of substances was investigated by measuring the oxygen uptake of the potato enzyme at p_H 8 and 20° in their presence.

Of these substances phloroglucinol, *p*-nitrophenol, α-naphthol, dimethylaniline, thiolacetic acid, cysteine, salicylaldehyde and benzyl alcohol acted as inhibitors, while the following substances had little or no effect: urethane, methylurethane, methyl, ethyl, amyl and octyl alcohols, glycerol, glucose, pyridine, piperidine, diethylamine, urea, *o*-cresol, salicylic acid, *p*-quinone, quinhydrone, guaiacol, phenolphthalein, formaldehyde, acetaldehyde, phenylglyoxal, diacetyl, vanillin, sodium cholate, sodium *n*-hexanoate, sodium *n*-octanoate, fluorescein, haematin, *p*-rosaniline, methylene blue, picric acid, sodium pyrophosphate, uric acid, amygdalin, benzonitrile and quinine sulphate. The substances tested were brought to p_H 8 by addition of dilute sulphuric acid or sodium hydroxide before being tested.

It was observed that the addition of glycine or aniline to the system reduced the inhibition by benzyl alcohol, resorcinol and phloroglucinol, but this phenomenon was not further investigated.

Secondary oxidation of o-phenylenediamine.

It has been shown qualitatively that the *o*-quinones formed by the oxidation of catechol derivatives can effect the secondary oxidation of gum guaiacum, the "Nadi Reagent" and certain amino-acids [Szent-Györgyi, 1925].

o-Phenylenediamine was found to be oxidised in a similar manner, and some experiments were carried out to show the concentration of catechol derivative required for the reaction to take place.

M/125 *o*-phenylenediamine (freshly recrystallised) underwent no measurable oxidation on shaking for 8 hours with the potato catechol oxidase at p_H 6·8. On the addition of 0·02 mg./ml. of adrenaline or dihydroxyphenylalanine to

the system, an easily measurable absorption of oxygen took place, and the solution darkened in colour owing to the oxidation of the *o*-phenylenediamine (Fig. 3).

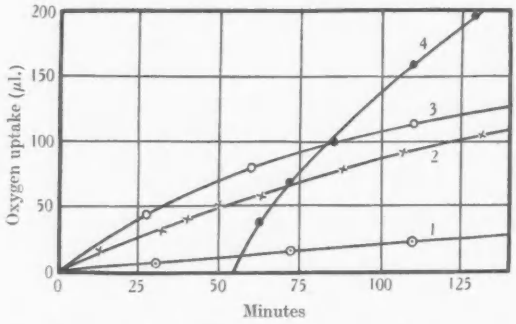

Fig. 3. In each experiment $M/125$ *o*-phenylenediamine, $M/50$ phosphate buffer p_H 6·8, and 0·54 c.u. potato catechol oxidase. Total volume 5 ml. Temperature 20°. 1. 0·02 mg./ml. adrenaline. 2. 0·1 mg./ml. adrenaline. 3. 0·06 mg./ml. dihydroxyphenylalanine. 4. 0·2 mg./ml. dihydroxyphenylalanine (dihydroxyphenylalanine in 4 added after 54 minutes).

Secondary oxidation of thiolacetic acid.

Since —SH compounds are known to be present in tissues which contain the catechol oxidase, it seemed probable that this system would be able to bring about the secondary oxidation of —SH compounds to the disulphides.

Attempts to demonstrate this oxidation by adding a solution of thiolacetic acid were unsuccessful, since the catechol oxidase was almost completely inhibited by —SH compounds. The —SH concentration was therefore reduced by adding to the system an aldehyde such as formaldehyde, with which —SH compounds form dissociable complexes [Pirie and Pinhey, 1929]:

$$R.CHO + R'.SH \rightleftharpoons R.CH(OH).SR'$$

It was found that the addition of formaldehyde greatly reduced the inhibition of the catechol oxidase and allowed a rapid oxidation of the thiolacetic acid to take place. The following figures give the results of four experiments at 20°. Total volume 5 ml. in each experiment.

Enzyme, 14·6 c.u./ml. (ml.)	—	0·3	0·3	0·3
$M/10$ phosphate buffer, p_H 6·0 (ml.)	1	1	1	1
$M/50$ Na thiolacetate (ml.)	1·5	1·5	1·5	1·5
30 % formaldehyde (ml.)	0·3	0·3	—	0·3
$M/5$ catechol (ml.)	0·2	—	0·2	0·2
Time of experiment (mins.)	240	164	270	120
Oxygen absorbed, μl.	0	0	94	343
Thiolacetate ($N/50$ iodine)	1·5	1·5	1·0	0·1

The catechol and formaldehyde present interfered with the direct iodimetric estimation of the thiolacetic acid at the end of the experiment. The procedure adopted was to precipitate the thiolacetic acid with lead acetate at p_H 5, centrifuge off and wash the precipitate with water. On treating with potassium iodide and sulphuric acid, the precipitate was decomposed. The lead iodide was centrifuged off and washed, and the thiolacetic acid in solution was titrated directly with $N/50$ iodine.

Oxidation of monohydric phenols.

In addition to effecting the oxidation of catechol derivatives, preparations of the catechol oxidase of potatoes also bring about the oxidation of monohydric phenols, such as *p*-cresol, *m*-cresol, phenol and tyrosine. The reaction is specific, since phenols such as guaiacol, *o*-cresol, salicylic acid, thymol, α-naphthol and in general those phenols which on oxidation are likely to give quinol derivatives rather than catechol derivatives, are not attacked [Onslow and Robinson, 1925; 1926; 1928; Pugh and Raper, 1927].

Raper [1928] and Pugh [1930] hold the view that one enzyme only is involved in the oxidation both of catechol derivatives and of monohydric phenols.

Onslow and Robinson [1928] observed that the rate of oxidation of monohydric phenols was greatly increased by the addition of a catechol derivative. They concluded that the oxidation of the monohydric phenols is a secondary oxidation, effected by a reaction product of the catechol oxidase system. In her earlier papers Onslow concluded that the intermediate substance which oxidises the monohydric phenol is an organic peroxide, but later she held that it might be either hydrogen peroxide or an *o*-quinone. She assumed that a second enzyme might catalyse the secondary oxidation of the monohydric phenols.

Pugh [1929] has shown that the addition of a trace of a catechol derivative or of an *o*-quinone also accelerates the rate of oxidation of monohydric phenols with the phenolase preparation from mealworms, but she does not accept Onslow and Robinson's explanation as to the mechanism. She has also shown that the oxidation of monohydric phenols is accelerated by the addition of a trace of hydrogen peroxide, but this might be due to the formation of *o*-quinone by the partial oxidation of the monohydric phenol by the hydrogen peroxide, catalysed by peroxidase present in the enzyme preparation.

Onslow and Robinson [1928] claimed that they had effected a partial separation of the system into two enzymes, but this was disputed by Pugh [1930].

Acceleration by catechol derivatives. In the present work Onslow and Robinson's colorimetric experiments on the effect of catechol derivatives on the oxidation of monohydric phenols have been confirmed and extended by quantitative measurements of the oxygen uptake, using the phenolase system of the potato. *p*-Cresol was used as a substrate in preference to tyrosine, in which the initial oxidation is followed by a series of reactions connected with the amino-acid grouping, which introduces an unnecessary complication.

The oxidation of *p*-cresol in the presence of crude potato juice was very rapid and showed a regular oxygen uptake. With the purified enzyme, on the other hand, the rate of oxidation was initially very slow, but increased autocatalytically to a high value (Fig. 4).

The initiation of the reaction was strongly accelerated by the addition of boiled potato juice, or of a trace of a catechol derivative (potato juice contains catechol derivatives) such as adrenaline, dihydroxyphenylalanine or catechol. The acceleration produced by even 0·4 mg./litre adrenaline could be measured.

As a possible explanation of the action of catechol derivatives Onslow and Robinson suggested that the hydrogen peroxide formed by their oxidation might cause the secondary oxidation of the monohydric phenols:

$$\text{Catechol derivative} + O_2 + (\text{catechol oxidase}) \rightarrow o\text{-quinone} + H_2O_2 \qquad \text{I.}$$

$$H_2O_2 + \text{monohydric phenol} + (\text{peroxidase}) \rightarrow \text{catechol derivative} + H_2O \qquad \text{II.}$$

On this view the oxidation of the monohydric phenol by hydrogen peroxide would be catalysed by peroxidase present in the enzyme preparation and would

give rise to a catechol derivative which would in turn be oxidised by the catechol oxidase. The process would be continuous and might be regarded as a type of chain reaction.

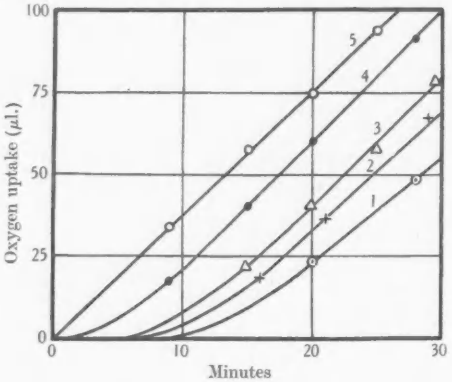

Fig. 4. In each experiment $M/125$ *p*-cresol, $M/50$ phosphate buffer p_H 6·8, and 2·4 c.u. potato enzyme. Total volume 5 ml. Temperature 20°. 1. No addition. 2. $M/250$ glycine. 3. 0·4 mg./litre adrenaline. 4. 0·5 ml. boiled potato juice. 5. 8 mg./litre adrenaline.

Against this view it was observed that the specimens of potato catechol oxidase purified by adsorption were almost free from peroxidase. On testing for peroxidase with gum guaiacum and hydrogen peroxide the enzyme solution gave only a faint blue coloration after standing for 10 minutes.

It was further observed that the addition of 0·3 % sodium pyrophosphate or saturation with pyrrole, which very strongly inhibit vegetable peroxidase [Elliott, 1932], had no appreciable effect on the rate of oxidation of *p*-cresol. It must be concluded that in the oxidation of monohydric phenols the peroxidase system is not primarily concerned.

The autocatalytic nature of the curve with the purified enzyme could be explained if during the purification a trace of inhibitor were accidentally introduced, and the reaction velocity slowly increased as the inhibitor was oxidised by the *o*-quinone produced during the reaction. This possibility was rendered improbable by the fact that the enzyme had the same properties when further purified by different methods, *e.g.* by adsorption, dialysis or precipitation with alcohol. Further, it was observed that when the enzyme was "activated" by the addition of a trace of catechol, it was found to be no longer activated if allowed to stand until the *o*-quinone formed had decomposed.

Inhibition of the oxidation of monohydric phenols. The most probable explanation of the action of catechol derivatives appeared to be Onslow and Robinson's suggestion that the monohydric phenols are oxidised by the *o*-quinones formed:

Catechol derivative + O_2 + (catechol oxidase) → *o*-quinone + H_2O_2 I.

Monohydric phenol + *o*-quinone + (dehydrase) → 2 mols. catechol derivative II.

Here again the reaction would be continuous, since reaction II regenerates the catechol derivative required for reaction I.

Attempts to test this view by showing that a trace of *o*-quinone could be added in the place of the catechol derivative were inconclusive, since *o*-quinones are very unstable in aqueous solution, and after standing only for a few minutes

the solution contained an appreciable amount of catechol derivatives [Willstätter and Müller, 1911]. The addition of sodium β-naphthoquinonesulphonate, an *o*-quinone derivative which is relatively stable in aqueous solution, produced only a slight acceleration.

This view of the action of *o*-quinones was therefore tested by adding to the system substances which combine with *o*-quinones. It was found that the oxidation of *p*-cresol or tyrosine was completely inhibited by 0·5 % *o*-phenylenediamine, *M*/1000 sodium bisulphite, 1 % potassium iodide or *M*/10 aniline for a period of over 5 hours, although the catechol oxidase was still active, since a rapid oxygen uptake occurred on adding catechol.

This inhibition by substances that combine with *o*-quinones gives additional evidence that *o*-quinones play an essential part in the oxidation of monohydric phenols. The view that one enzyme catalyses the direct oxidation of both catechol derivatives and monohydric phenols by molecular oxygen [Raper, 1928; Pugh, 1930] does not explain the extreme sensitivity of the latter, but not the former, reaction to traces of *o*-quinones and to substances which combine with *o*-quinones.

The instability of the *o*-quinones in solution makes it difficult to obtain direct experimental proof, but the oxidation potentials of the *o*-quinones are sufficiently high for the oxidation of the monohydric phenols, and the available evidence is consistent with the view that this oxidation takes place.

Summary.

1. Catechol oxidase preparations from *Syringa vulgaris*, *Sambucus nigra*, *Agaricus campestris*, *Polyporus hispidus*, *Tenebrio molitor* and potato were compared by testing the extent to which they were inhibited by varying concentrations of resorcinol. Preparations of different origin showed marked specific differences in their behaviour. An improved method was obtained for preparing stable preparations of the catechol oxidase of potatoes.

2. Conditions were found under which the catechol oxidase system of potatoes effected the secondary oxidation of *o*-phenylenediamine and of thiolacetic acid.

3. Measurements of the rate of oxidation of *p*-cresol by the catechol oxidase of potatoes showed the reaction to be strongly accelerated by substances which produce *o*-quinones, and strongly inhibited by substances which combine with *o*-quinones. The reaction would therefore appear to be essentially a secondary oxidation in which the formation of an *o*-quinone is involved, rather than a direct oxidation of *p*-cresol by the enzyme.

The author wishes to thank Prof. Sir Frederick Gowland Hopkins and also Mrs Adair for their interest and advice.

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CXXX. THE ACTION OF CRYSTALLINE VITAMIN B_1 ON THE RESPIRATION OF POLYNEURITIC TISSUES *IN VITRO*.

By ROBERT HENRY STEWART THOMPSON[1].

From the Department of Biochemistry, Oxford.

(*Received May 1st, 1934.*)

THE connection of vitamin B_1 with tissue oxidation has been considered for many years, and recent work has strongly suggested that this vitamin is intimately associated with the oxidation of carbohydrates. Gavrilescu and Peters [1931] showed that the oxygen uptake of minced polyneuritic pigeon's brain tissue in the presence of added glucose was significantly lower than that of the normal tissue. More recently [Gavrilescu *et al.*, 1932] this has been shown to be the case in the presence of added lactate as well, an average diminution of 33 % being found for cerebrum, and 45 % for the optic lobes; in the presence of succinate however there is no difference between the levels of oxidation of normal and avitaminous tissues, while the residual uptake, *i.e.* without added substrate, also shows no significant difference. The addition of small amounts of crystalline vitamin B_1 restores the oxygen uptake of the avitaminous tissue in the presence of lactate almost to the normal level. It is concluded that this *in vitro* effect of the vitamin on the respiration of brain tissue is specifically connected with the glucose-lactate enzyme systems, and this action is now referred to as the catatorulin effect of the vitamin [Passmore *et al.* 1933].

It seems to be well established therefore that there exists a specific defect in the oxidative mechanism of the brain in B_1 avitaminosis, but with regard to the other tissues the position is not at present clear. The literature concerning the level of respiration in normal and avitaminous tissues other than brain is confused and contradictory, as may be seen from Westenbrink's review of the subject [1932, 1]. In the fifteen investigations reviewed the only positive results, as Westenbrink has pointed out, which survive statistical examination are those of Ahlgren [1923] on minced pigeon muscle and of Tsukamoto [1928] on pigeon liver slices[2].

Galvao and Cardoso [1932] investigated, by the methylene blue method, the reducing powers of normal and avitaminous tissues and of tissues from animals in a state of inanition. They found that the reducing power of muscle tissue from animals suffering from inanition was lower than that of the normal tissue, but that there was no lowering of the reducing power of muscles from polyneuritic animals when excessive loss of weight was prevented by forced feeding on polished rice; in the case of liver they found no difference even between normal animals and animals in a state of inanition.

[1] Senior Demy of Magdalen College.

[2] For further discussion of the experiments of Abderhalden and Vlassopoulos [1931] see Gavrilescu *et al.* [1932].

Westenbrink, impressed with the fact that Tsukamoto's positive results had been obtained with liver slices in contrast with the minced tissues which had been used by other workers, has recently [1932, 2] repeated the experiments of Tsukamoto, but has been unable to find any decrease in the oxygen uptake of liver slices either from pigeons or rats.

It is perhaps worthy of mention that in none of the experiments reviewed by Westenbrink, except those of Drummond and Marrian [1926] on the reducing powers of the tissues for methylene blue, nor in Westenbrink's own experiments, was any added substrate used, the tissue being placed merely in Ringer solution or in a Ringer buffered by phosphate or bicarbonate.

In view of the conflicting results obtained in the comparison of the tissue respiration of normal and avitaminous animals, the work of Peters and his colleagues on the catatorulin effect of vitamin B_1 in polyneuritic pigeon's brain tissue seems to provide a more decisive method for determining the presence of such an oxidative lesion in other avitaminous tissues. By analogy with brain, the addition of vitamin B_1 to suspensions of these tissues respiring in lactate should result in an increased oxygen uptake, if such an oxidative lesion exists. Such a procedure avoids the controversial question of the level of tissue respiration in vitamin B_1 deficiency, and also has the advantage that it is less dependent on the general state of nutrition of the animal. The following experiments therefore were primarily undertaken not in the hope of settling whether there was a specific lowering of cell-respiration in polyneuritic tissues, but to determine whether added vitamin B_1 showed any catatorulin activity in the tissues of polyneuritic pigeons other than the brain.

Method.

The method employed has been to kill the birds by guillotining. The organ to be investigated is then quickly dissected out and removed from the body; any surrounding connective tissue is carefully dissected away and the organ gently wiped free from blood with filter-paper. In dissecting out the kidney it has been found most convenient to pluck the back of the abdomen and then to remove the entire piece of the bony posterior abdominal wall in front of which the kidney lies: by this means the kidney can be removed with the least amount of bleeding. Muscle, heart, liver and kidney have been investigated, the muscle employed being the pectoralis major.

In all cases except where subsequently mentioned the tissues have been finely chopped up with scissors: as far as was possible to judge the tissues were minced to the same extent each time. In those experiments in which sections were used the tissue was sliced free-hand with a razor moistened with the tissue fluid only, as recommended by Harrison [1933].

The oxygen uptake was measured in bottles of the Barcroft-Dixon type containing 3 ml. of Ringer-phosphate solution at p_H 7·3 (made up according to the usual formula used in this laboratory) to which neutralised sodium lactate, prepared from recrystallised zinc lactate, had been previously added in the amount required to make the final concentration of lactate 0·033 *M*. To the bottles containing vitamin 2·8 ml. of Ringer-phosphate-lactate solution were added before the addition of the tissue; the subsequent addition of a further 0·2 ml. of the medium containing 1–2 γ of the crystalline vitamin [Kinnersley *et al.* 1933] bringing the final volume up to 3 ml. The bottles were weighed before and after the addition of the tissue; after the second weighing the tissue

was finely broken up with a flat-ended glass rod and the vitamin added to those bottles where required. Rolls of filter-paper moistened with 2*N* KOH were used to absorb the CO_2. The bottles were then twice evacuated to 70 mm. and filled with oxygen. The temperature of the bath was 38°.

All the avitaminous birds, which were fed according to the usual procedure adopted in this laboratory, showed well-marked symptoms of head-retraction at the time of use. The amounts of tissue employed in each case varied with the different tissues on account of differences in their rates of oxygen consumption: the approximate average amounts per bottle were 70 mg. for kidney, 100 mg. for muscle and liver, and 130 mg. for heart. All the values given are the averages of duplicate estimations, the agreement being in nearly every case within 7 %.

Results.

It was found that with avitaminous kidney the addition of the vitamin produced in all cases a large and striking increase in the oxygen uptake measured over four successive half-hour periods, but with muscle, heart and liver the effect when present was always small and not actually statistically significant.

The results obtained with avitaminous muscle, heart and liver, with and without the addition of vitamin are shown in Table I.

The oxygen uptakes are expressed in microlitres of oxygen per g. of tissue per hour over four successive half-hour periods: this method of expression has been used in order to render the results more comparable with those of other workers on this problem; for those who prefer the modern nomenclature 2000μl./g./hour$=Q_{O_2}$ 10 approximately. It can be seen from this table that the oxygen uptake of these organs is little affected by the addition of the vitamin: in the muscle and heart experiments the bottles containing vitamin had in some cases a lower uptake than the controls, and in those cases where the vitamin did bring about an increase, this increase was always small, reckoned either as the excess amount of oxygen taken up in μl. per g. of tissue per hour or as the percentage rise over the control, and was not statistically significant[1]. In the case of the liver the addition of the vitamin produced in every case a small increase in the oxygen uptake, but here also though the actual amount of oxygen taken up was greater than in the case of muscle or heart, the percentage increase was of the same order and was again not statistically significant except possibly for the fourth half-hour period. Since the vitamin effect, when present, was in every case so slight, and in view of the fact that statistical examination of the results shows that the increase is definitely not significant, it was thought unnecessary to do more than five of these experiments on each tissue. Four additional experiments were done using slices of liver, but no effect was obtained by this method.

Table II shows the results obtained with avitaminous kidney with and without the addition of vitamin. As in the previous experiments the amount of vitamin added was 1–2 γ per bottle. It may be seen that with kidney the addition of the vitamin produced in every case a large increase in the oxygen uptake, obviously of quite a different order from the effects obtained with the other tissues, amounting in the fourth half-hour period to 885μl., or 58 % of the oxygen uptake without added vitamin.

[1] The significance of the results obtained in this research was determined by means of Fisher's "t" test.

Table I. *Avitaminous heart, muscle and liver.*

O_2 uptake (μl. O_2/g./hour).

Heart.

Exp.		Periods (hours) 0–½	½–1	1–1½	1½–2
8.	L.	850	430	345	255
	L. + V.	835	460	412	265
11.	L.	725	447	277	182
	L. + V.	772	464	274	189
12.	L.	837	475	252	193
	L. + V.	872	506	351	202
14.	L.	715	341	178	165
	L. + V.	804	379	213	158
37.	L.	705	335	179	156
	L. + V.	695	405	214	167
Average	L.	765	405	245	190
	L. + V.	795	445	295	195
Average increase		+30	+40	+50	+5

Muscle.

5.	L.	2555	794	257	—
	L. + V.	2410	937	305	—
35.	L.	750	415	294	232
	L. + V.	770	460	262	179
37.	L.	780	300	146	76
	L. + V.	700	310	140	116
38.	L.	1415	480	213	118
	L. + V.	1535	510	222	138
39.	L.	545	193	114	95
	L. + V.	515	202	154	105
Average	L.	1210	435	205	130
	L. + V.	1190	485	215	135
Average increase		−20	+50	+10	+5

Liver.

30.	L.	1105	800	597	442
	L. + V.	1185	868	667	506
34.	L.	1265	890	650	—
	L. + V.	1290	890	660	—
35.	L.	2200	1330	905	675
	L. + V.	2210	1340	1020	760
37.	L.	1465	1090	715	585
	L. + V.	1765	1205	830	708
38.	L.	1870	1230	900	600
	L. + V.	1960	1280	980	715
Average	L.	1580	1070	755	575
	L. + V.	1680	1120	830	670
Average increase		+100	+50	+75	+95

Table II. *Avitaminous kidney.*

O_2 uptake (μl./g./hour).

Exp.	Periods (hours) 0–½	½–1	1–1½	1½–2
11. L.	2120	2060	1845	1485
L. + V.	2335	2500	2385	2220
12. L.	2100	2075	1760	1525
L. + V.	2450	2480	2405	2340
14. L.	2355	2275	1885	1555
L. + V.	2735	2935	2735	2660
19. L.	2265	2120	1790	1585
L. + V.	2820	2780	2790	2630
24. L.	1870	1875	1725	1475
L. + V.	2340	2140	2490	2215
34. L.	2365	2130	1840	1575
L. + V.	2630	2650	2515	2450
Average L.	2180	2090	1810	1535
L. + V.	2550	2580	2550	2420
Increase (μl. O_2)	370	490	740	885
% increase	17	23	41	58

It is also noteworthy that whereas the respiration of the avitaminous kidney *in vitro* falls off steadily, the addition of the vitamin maintains the respiration at an almost constant level. In view of this catatorulin effect of the vitamin on the kidney as well as on the brain it is of interest to compare the rates of respiration of normal and avitaminous kidney tissue in the presence of lactate. The results obtained with normal kidney without added vitamin are shown in Table III.

Table III. *Normal kidney.*

O_2 uptake (μl./g./hour).

Exp.	Periods (hours) 0–½	½–1	1–1½	1½–2
13	3120	3455	3085	3225
17	2440	2510	2425	2485
18	2830	2865	2680	2860
27	2470	2460	2775	2265
28	3075	3145	2965	3150
43	3570	3345	3475	3265
Average	2920	2965	2900	2875

It will be seen that the respiration of normal kidney, like that of the avitaminous kidney in the presence of added vitamin, maintains itself at a fairly constant level (Fig. 1). Statistical examination of these figures also shows that the level of oxygen uptake of the avitaminous kidney is significantly lower than that of the normal, while Table III shows that the lowered level of respiration of the avitaminous kidney is appreciably restored by the addition of the vitamin. Four experiments were done to determine whether added vitamin had any effect on the respiration of normal kidney in the presence of lactate; it was found that there was no effect.

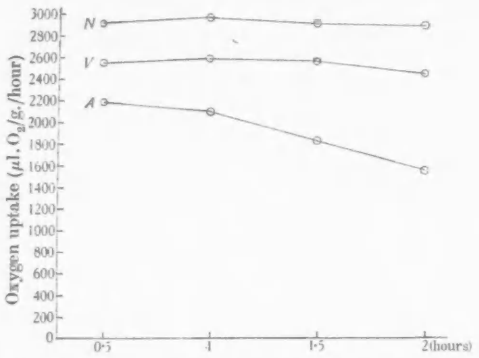

Fig. 1. Mean rate of oxygen uptake of minced kidney in presence of lactate.

N = normal; *A* = avitaminous; *V* = avitaminous + vitamin.

Discussion.

It seems justifiable to conclude from the results of this research that the addition of vitamin B_1 to muscle, heart or liver tissue from polyneuritic pigeons has no significant effect on the oxygen uptake *in vitro*, but that it shows a marked catatorulin effect on polyneuritic kidney respiring in the presence of lactate.

As already pointed out the primary object of this research was to investigate, not the rate of respiration of normal and avitaminous tissues, but the catatorulin effect of the vitamin in polyneuritic tissues other than brain. But in view of the discovery of this striking effect on polyneuritic kidney, it seemed essential to determine whether the extra oxygen uptake produced by the added vitamin was a restoration of a lowered level of respiration to the normal level. The results show that, as in the case of brain, the respiration of the polyneuritic kidney *in vitro* in the presence of added lactate is markedly lower than that of the normal tissue, the lowering becoming more pronounced in the third and fourth half-hour periods. There exists therefore in the kidneys of polyneuritic pigeons in opisthotonus some oxidative lesion which is capable of being largely repaired *in vitro* by the addition of vitamin B_1. No experiments have yet been done to decide the stage in avitaminosis at which the kidney lesion commences.

It is possible that this kidney lesion may be connected in some way with the oedema which is sometimes present in polyneuritic birds, but it is certain that other factors must also be concerned, since no obvious oedema was present in any of the birds used during this research.

Summary.

1. The respiration of minced muscle, heart, liver and kidney tissues from polyneuritic pigeons has been studied in the presence of lactate with and without the addition of small amounts of vitamin B_1.

2. The addition of the vitamin to polyneuritic muscle, heart or liver has no significant effect on the oxygen uptake of these tissues *in vitro*, but in the case of polyneuritic kidney the addition of the vitamin brings about a large and statistically significant increase in respiration.

3. The respiration of the polyneuritic kidney in the presence of lactate is significantly lower than that of normal kidney; the addition of the vitamin largely restores this defect.

I wish to express my sincere gratitude to Prof. Peters for his constant interest and advice during the course of this work. I am also indebted to Mr H. W. Kinnersley for supplying the vitamin concentrates used, and to Mr R. B. Fisher for assistance with the statistical examination of the results.

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CXXXI. PYRUVIC ACID AS AN INTERMEDIARY METABOLITE IN THE BRAIN TISSUE OF AVITAMINOUS AND NORMAL PIGEONS.

By RUDOLPH ALBERT PETERS and
ROBERT HENRY STEWART THOMPSON.

From the Department of Biochemistry, Oxford.

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The recent discovery of a pyruvate reaction in lactate solutions in which avitaminous pigeon's brain has respired *in vitro* for 2 hours [Peters and Sinclair, 1933] is of interest in relation to the action of vitamin B_1, because addition of this reduces the amount of the abnormal substance (for references see Gavrilescu *et al.* [1932] and Meiklejohn *et al.* [1932]). More generally, identification of pyruvate in these circumstances lends support from an unexpected source to the recent conceptions of this substance as a normal intermediary in the metabolism of carbohydrate in the animal (see especially Embden *et al.* [1933] and Meyerhof and Kiessling [1933]). Otherwise these views depend entirely upon work with added sulphite or fluoride. There is also involved the even more fundamental problem of synthesis of carbohydrate from lactic acid, since the indication is that vitamin B_1 is related to the oxidative synthesis of lactate. With this idea in mind, we have devoted attention in the main to phenomena occurring in lactate solutions, as we are more likely so to approximate to conditions favouring the synthesis. The experiments described in this paper have been made with minced brain and in phosphate solutions to facilitate manipulations and to obtain better duplicate control. The conditions are frankly artificial and narrowed for the study of the one point, but it is most unlikely that qualitative errors have been imposed by our conditions. The presence of the substance reacting like pyruvate is equally induced by sections of avitaminous brain and was found by Sinclair (personal communication) to occur with bicarbonate solutions.

We have proved that the nitroprusside-reacting substance is actually pyruvic acid and have obtained quantitative data relating extra pyruvate disappearance in presence of vitamin to extra oxygen uptake to find out whether pyruvate disappears as a direct or indirect effect of the vitamin[1].

Experimental.

Pyruvic acid is known to bind bisulphite. It can be readily shown that bisulphite-binding substances (B.B.S.) (*a*) do not appear during the respiration of normal brain tissue (*b*) appear during that of polyneuritic tissue, and (*c*) disappear with addition of vitamin. Bisulphite-binding substances however do appear during the respiration of normal brain tissue in the presence of iodoacetate.

[1] A preliminary account has appeared in *J. Physiol.* **81**, 22 P.

Unless otherwise stated the minced[1] brain tissue from normal or polyneuritic pigeons (with opisthotonos symptoms) has been shaken in phosphate Ringer-lactate or pyruvate solution at p_H 7·3 [see Passmore *et al.*, 1933] in differential manometers filled with oxygen: in all cases anhydrous sodium pyrophosphate (brought to p_H 7·3 with HCl) was added in a concentration of 0·0094 *M* per bottle [Peters and Sinclair, 1933]. At the end of the respiration period (usually 2 hours) at 38° to each cooled bottle was added 0·9 ml. of 25 % trichloroacetic acid. After standing 1 hour the contents were filtered and the bottles washed out with 2 ml. of 5 % trichloroacetic acid.

Table I.

Bisulphite-binding substances (*B.B.S.*).

Results are expressed in ml. *N*/100 I per g. tissue
(1 ml. = 0·44 mg. pyruvic acid).

I. Residual.

Exp.		Initial	R.P. 2 hours	R.P. + V. 2 hours	Difference produced by vitamin
533	Normal	—	1·18	—	—
562	Avitaminous	—	1·64	1·60	0·04
663	,,	—	2·08	1·69	0·39
535	,,	1·17	—	—	—
564	,,	—	2·80	1·74	1·06
565	,,	—	1·98	1·74	0·24
		Average	**2·12**	**1·69**	**0·43**

II. Lactate.

Exp.		Initial	L. 1 hour	L. 2 hours	L. + Iodo.	L. + Fl.
545	Normal	—	—	2·75	—	2·72
531	,,	0·92	2·32	(3·30) (3·34)	—	—
533	,,	—	—	2·87	—	—
534	,,	—	—	3·10	—	—
541	,,	—	—	3·69	7·25 (0·0018 *M*)	3·04
592	,,	—	—	4·23	8·2 (0·0005 *M*)	3·8
			Average	**3·33**	**7·72**	**3·19**

(Iodoacetic acid concentration in brackets. Fluoride 1 mg./ml. NaF (0·024 *M*).

Avitaminous.

Exp.	L. 1 hour	L. + V. 1 hour	Difference	L. 2 hours	L. + V. 2 hours	Difference	L. + Fl. 2 hours
535	6·88	5·5	1·38	10·2	7·14	3·06	5·8
539	—	—	—	9·37	5·63	3·74	—
537	5·96	3·78	2·18	7·16	3·93	3·23	—
551	6·6	5·4	1·20	8·95	6·58	2·37	—
553	—	—	—	8·85	6·41	2·44	—
554	—	—	—	9·30	5·23	4·07	—
557	—	—	—	9·88	6·03	3·85	—
558	—	—	—	10·0	5·10	4·90	—
560	—	—	—	11·10	5·33	5·77	—
569	—	—	—	7·45	4·69	2·76	—
580	—	—	—	9·8	5·28	4·52	5·33
Average	**6·48**	**4·89**	**1·59**	**9·28**	**5·58**	**3·70**	**5·56**

[1] In unpublished results we have found that a large quantitative difference may be induced in respiration by variations in temperature of mincing. The data as to temperature have not been included in this paper, because no qualitative effect seems to have been produced upon the quotient studied.

The combined filtrates were treated with N NaOH to bring to p_H 2·0 and made up with water to a final volume of 15 ml. for each bottle containing originally 3 ml. of Ringer *plus* brain tissue. 5 ml. of this solution were then treated with 0·5 ml. of M $NaHSO_3$ and allowed to stand for 15 min. [Clift and Cook, 1932]. Excess bisulphite was removed with $N/10$ and then $N/100$ iodine; 5 ml. of saturated $NaHCO_3$ were added and the liberated bisulphite was titrated with $N/100$ iodine. The results are given in Tables I and I A.

Table I A.

Abstract of results (averages).

	Initial	R.P. 2 hours	R.P.+V. 2 hours	Diff.	L. 1 hour	L.+V. 1 hour	Diff.
Residual.							
Normal (1)	—	1·18	—	—	—	—	—
Avitaminous (5)	1·17	2·12	1·69	0·43	—	—	—
Lactate.							
Normal (6)	0·92	—	—	—	2·32	—	—
Avitaminous (11)	—	—	—	—	6·48	4·89	1·59

	L. 2 hours	L.+V. 2 hours	Diff.	L.+Fl. 2 hours	L.+Fl.+V. 2 hours	Diff.	L.+Iodo.	L.+Gl.	L.+Gl.+Iodo.
Lactate.									
Normal (6)	3·33	—	—	3·19	—	—	7·72	3·18	5·67
Avitaminous (11)	9·28	5·58	3·70	5·56	3·90	1·66	—	—	—

Results in Table I are the mean of duplicate estimations; they agree within ±2 % for L. (avit.), (±0·01 ml. iodine for the equivalent of 1·0 ml. bottle contents). For smaller amounts of pyruvate the error is proportionately greater. V. = in all cases, 2γ crystalline vitamin B_1 hydrochloride per Barcroft bottle [Kinnersley *et al.*, 1933].

Substances combining with bisulphite are present in very small amounts in normal brain and increase slightly during respiration in lactate (not more than 0·4 mg. per g.). The amounts formed with avitaminous brain are of a different order (ten times as much); they decrease 39 % with added vitamin.

A comparison of experiments in Ringer-phosphate only, with those in lactate show a slight increase of B.B.S. with lactate in normal, but a large increase for avitaminous brains. Hence lactate is essential for the formation of the B.B.S. which are decreased in amount by added vitamin. Before proceeding further, it is necessary to know how much of the B.B.S. is really pyruvic acid. The method of Clift and Cook [1932] of heating in alkali to remove substances other than pyruvic acid did not prove satisfactory in our hands, and could not be so certain as isolation of the 2:4-dinitrophenylhydrazone [Case, 1932].

Case's method involves the following steps:

1. Addition of 2:4-dinitrophenylhydrazine to the trichloroacetic acid solution.
2. Extraction of mixed hydrazine and hydrazones with ethyl acetate.
3. Neutralisation with $CaCO_3$.
4. Evaporation and extraction with toluene.
5. Extraction with Na_2CO_3.
6. Acidification and extraction with ethyl acetate.
7. Evaporation to dryness.
8. Estimation in solution in alcoholic potash.

This has been modified as follows. Stage 1. Same; 5 ml. 1 % hydrazine in 2N HCl added to 20·0 ml. trichloroacetic acid solution.

2. Same; at least 3 extractions, and often more.

3. Successive extractions with alkaline phosphate, 1/3 vol. The first extraction with saturated alkaline phosphate (Na), and subsequent with *M*/15.

4. Acidification with HCl and extraction with ethyl acetate.

5. Repeat stage 3.

6. Repeat stage 4, making acid to p_H 2·5 approx.

7. As Case.

8. As Case.

We found that our extracts did not give good results by Case's method unmodified. Since the pure hydrazone of pyruvic acid was found to be completely extracted by alkaline phosphate we proceeded as in stages 3 and 4, continuing the extraction with alkaline phosphate until fresh extracts were colourless, or showed only a faint brown-yellow colour, due to traces of hydrazine. With this exception other stages were repeated until colourless. The trace of hydrazine carried over into 4 is removed almost completely in 5 and 6. The combined ethyl acetate solutions from 2–3 bottles (equivalent to 200–300 mg. tissue) are conveniently made up to 5–6 ml. 1–2 ml. (Ostwald pipette) can be evaporated in a small measuring flask to 0·1 ml., cooled and made up to the mark with the alcoholic potash. Standards containing 0·5 mg. per 1·0 ml. ethyl acetate have been used for comparison ($\equiv$0·15 mg. pyruvic acid). The 0·1 ml. ethyl acetate left assists solution in the alcoholic potash and minimises the risk of hydrolysing the hydrazone. It appears to have no influence upon the determination provided that the estimation is performed reasonably soon.

The method has the great advantage that heating is always avoided up to stage 6, and if necessary after this by evaporation *in vacuo*. Hence unstable hydrazones such as that of oxaloacetic acid [Clift and Cook, 1932] should be detected more easily. Stages 2–6 for 4 estimations take approximately 4 hours.

Stage 7 reaches a high accuracy with 0·5 mg. or even less of hydrazone. Stages 2–6 give a good recovery of pyruvic acid 2:4-dinitrophenylhydrazone added at stage 2 to the combined ethyl acetate extracts. Examples are given in Table II.

Table II. *Recoveries.*

1. Pyruvic acid 2:4-dinitrophenylhydrazone added at stage 2. Final ethyl acetate phase made up to 6·0 ml. approx. and 2·0 ml. taken for analysis in all except *c* (3·0 ml.). 1·0 mg. $\equiv$0·328 mg. pyruvic acid.

	Added mg.	Found mg.	Corr. mg.
(*a*)	1·0	1·02	1·0
(*b*)	1·0	1·02	1·0
(*c*)	1·0	1·03	1·0
(*d*)	1·5	1·49	1·48

2. Pyruvic acid added at stage 1.

	Added mg.	Found mg.	Corr. mg.
(*e*)	0·492	0·494	0·488
(*f*)	0·492	0·496	0·490
(*g*)	0·984	0·984	0·981
(*h*)	0·984	0·960	0·957

As stated previously a trace of hydrazine accompanies the hydrazone, making a difference of approx. 0·01 mg. hydrazone (0·003 mg. pyruvic acid) per 1·0 ml. of final phase taken. This can be allowed for as a blank, if desired. Our slight modification is presumably more specific in its extraction than the Na_2CO_3 stage, but we have not investigated this.

Table III gives the results of estimations by the modified Case 2:4-dinitrophenylhydrazone method upon solutions from some of the experiments given in Table I. The experiments are given in order and probably increase in accuracy;

Table III. *Substrate lactate. Pyruvic acid, estimated by 2:4-dinitrophenylhydrazone method. mg./g.*

Exp.	L.	L. + V.	L. − (L. + V.)	L. % *	L. + V. % *	Diff. O_2 uptake	µl./ mg. P.A.	Remarks
554	3·32	1·66	1·66	81	72	1345	815	M.P. hydrazone low, 193°
557	2·90	1·68	1·22	67	63	962	790	M.P. 216°
558	3·67	1·57	2·10	84	69	1010	480	M.P. low, 204°
560	3·30	1·37	1·93	68	58	1115	580	(No pyrophosphate)
569	3·31	1·79	1·52	102	88	1162	765	
580	3·88	1·89	1·99	90	82	1020	515	
Average	**3·40**	**1·66**	**1·73**	**82**	**72**	**1102**	**657**	

* % of B.B.S. estimated as pyruvic acid 2:4-dinitrophenylhydrazone.

Exp. 590. Samples of avitaminous cerebrum in bottles as follows: 4 contained L. only, 4 L. + V. All were treated similarly, but two of each were removed from the bath after the preliminary shaking period, cooled and treated with trichloroacetic acid. The remainder were shaken 1 hour.

	min.	Pyruvic acid: As B.B.S.	Pyruvic acid: As 2:4-dinitro.	% as pyruvic acid	Weight tissue	− P.A./g.	Diff. P.A.	O_2/g.
L.	12	0·185	0·075	41	0·174	0·43	—	—
L.	60	0·502	0·398	78	0·205	1·91	1·48	1710
L. + V.	12	0·157	0·068	50	0·215	0·32	—	—
L. + V.	60	0·290	0·218	72	0·194	1·08	0·76	2070

Diff. P.A. = 1·48 − 0·76 = 0·72 mg.
Diff. O_2 = 360 µl.
O_2/P.A. = 500.

Note. Single estimations only were performed in these experiments owing to the small amount of pyruvic acid present.

in all cases L. and L. + V. have been extracted side by side. The last experiment (590) is given separately; an attempt was made to estimate the influence of the initial period upon the pyruvic acid disappearance, when there is no estimation of oxygen. The ratio $+O_2/-$pyruvic acid is within the limit for the others. Pyruvic acid evidently forms a large proportion of the B.B.S. and disappears in presence of added vitamin. Some 20 % of B.B.S. are not pyruvic acid, and this proportion increases as the result of the vitamin action. From this it may be inferred that one of the substances into which pyruvic acid is transformed is a non-acidic substance containing an aldehyde or ketone group.

In Exps. 551, 557, after one or two further purifications by repetition of stages 5 and 6, pure pyruvic acid hydrazone separated upon evaporation of the ethyl acetate phase, M.P. 216°, which showed no alteration in a mixed M.P. Traces of other hydrazones which give a red colour with alcoholic potash often accompany the pyruvic acid hydrazone, giving this a red tint and a lower M.P.; the amount of such substances is small, so that there seems no doubt that the main substance concerned is pyruvic acid.

Pyruvate as substrate.

The conclusion that the vitamin catalyses disappearance of pyruvate as well as increased O_2 uptake can be confirmed by substitution of pyruvate for lactate in the respiring system. This is in striking contrast to lactate, extra disappearance of which was not shown to take place in presence of vitamin [Meiklejohn, 1933].

In the experiments recorded in Table IV (A, B) the amounts of pyruvate present in the Ringer-phosphate solution were 5·0 mg. approx. (0·019 *M*).

Table IV. (A) *Pyruvate, titrated as B.B.S.*

(Data for sum of duplicate bottles.)

	P.					P.+V.			
Exp.	Initial pyruvate mg.	Weight tissue g.	Pyruvate 2 hours mg.	Pyruvate (mg.) disappearing per g.	O_2 uptake per g. 2 hours μl.	Weight tissue g.	Pyruvate 2 hours mg.	Pyruvate mg./g.	O_2 uptake per g. 2 hours μl.
562	10·1	0·2302	8·9	5·2	1220	0·2192	8·1	9·15	2450
563	[9·91]	0·220	8·9	[4·6]	1270	0·1944	8·7	[6·25]	2290
564	10·86	0·2370	9·55	5·52	1350	0·1787	9·6	7·06	2050
565	[10·36]	0·1983	9·45	[4·6]	2180	0·1589	9·2	[7·3]	3165
567	11·5	0·1966	9·95	7·9	1700	0·2112	9·4	9·95	2540
582	10·3	0·1975	9·8	2·53	1635	0·1850	9·45	4·6	2855
588	11·95	0·1664	11·55	2·4	2170	0·1697	10·83	6·6	3595
590	11·95	0·1971	11·17	3·95	1785	0·2049	10·3	8·05	2870
567*	11·5	0·2025	10·2	6·42	1275	0·2233	9·2	10·3	2850

* With lactate present also.

(B) *Pyruvate, estimated by hydrazone method*.*

	P.		P.+V.	
Exp.	Pyruvate 2 hours mg.	% B.B.S.	Pyruvate 2 hours mg.	% B.B.S.
563	8·4	94·5	8·15	94
564	9·2	96	8·4†	87·5†
565	8·65	91·5	8·25	90
567	9·37	94	9·3	99
567‡	9·75	95·5	8·9	97
582	9·08	92·5	8·56	90·5
588	10·7	92·5	10·11	93·5

* Mean of duplicate observations agreeing within 2 %, in exps. 567, 582, 588. Remainder single observations.

† Some known to have been lost. ‡ With lactate present also.

Table IV (B) shows that there is much more satisfactory agreement between the B.B.S. by titration and the hydrazone estimations than for lactate. A summary of the essential differences is given in Table V.

Table V. *Substrate pyruvate. The relation between pyruvate disappearing and O_2 uptake due to added vitamin B_1 (differences only).*

Exp.	Pyruvate mg./g.	O_2 uptake ml./g.	O_2/P.A.
562	3·72	1230	330
563	1·55	1020	658
564	1·45	700	482
565	2·53	985	389
567	1·93	840	435*
582	1·95	1220	625
588	3·95	1425	361
590	3·95	1085	275
Average	**2·63**	**1063**	**443**

* With lactate also present 380.

A point needing discussion arises in connection with some of the data in Table III. If the amounts of tissue are approximately equal the initial amount of pyruvate need not be known; if not, error may be introduced. Expressed mathematically:

If c = initial concentration of pyruvate,
a_1, a_2 = amounts present after time t, in pyruvate and pyruvate-vitamin respectively,
b_1, b_2 = weights of tissue (mg.),
k = constant,
d = difference wanted, all being in suitable units,
we have made the general assumption that

$$(c-a_1)=kb_1, \text{ etc.} \qquad \text{.....(1),}$$

$(c-a_1)\frac{100}{b_1}$ and $(c-a_2)\frac{100}{b_2}$ = disappearance of pyruvic acid per 100 mg. of tissue for P. and P. + V. respectively.

Hence

$$d=\frac{100}{b_1.b_2}[c\,(b_2-b_1)+a_2b_1-a_1b_2] \qquad \text{.....(2).}$$

Unless $b_1=b_2$, it is necessary to know c.

In most experiments c is known; in Exps. 563, 566, its value was doubtful owing to changes which pyruvic acid appears to undergo at slightly alkaline reaction. Clift and Cook [1932] heat in alkaline solution to remove bisulphite-binding compounds other than pyruvic acid, and we have found that over-neutralisation with NaOH has little effect upon the pyruvate provided that the solution is dilute. But direct addition of N NaOH to pyruvic acid, a procedure adopted in some experiments to minimise volume, may be attended by 50 % changes in bisulphite-binding power. This was not realised at first.

In Exps. 563 and 565, where low values were obtained for the titration of bisulphite-binding substances, we have substituted for c by use of the equation

$$(c-a_1)=k_1b_1 \qquad \text{.....(3).}$$

Other known errors in estimating the final quotient lie in the initial 12 minute period in which pyruvic acid disappears and the oxygen uptake is not estimated. In general this will not be serious, as it affects both P. and P. + V., and the oxygen uptakes differ less at first than afterwards.

The extra amounts of pyruvic acid disappearing are of the same order though larger than those with lactate, though the extra oxygen uptakes are very similar in the two sets of experiments, *i.e.* the catatorulin effect is not changed by the substrate. Addition of lactate to pyruvate also caused no change in behaviour in Exp. 567. The consistent vitamin effect with pyrophosphate present is in striking contrast to the earlier work of Meiklejohn *et al.* [1932], who found in absence of pyrophosphate an abnormally lowered respiration with pyruvate and a most variable vitamin effect. From this it was concluded that pyruvate was not a stage in the oxidation of lactic acid, and that another factor was needed to secure pyruvate oxidation. That the failure to interact was not due to blockage by excess lactate in the avitaminous brains was proved by the use of iodoacetic acid. The lowered respiration has been a constant feature of these experiments, but the consistency of the catatorulin effects indicates that pyrophosphate takes the place of the missing factor.

Pyruvic acid is the first organic substance which has been proved to disappear *in vitro* as the result of action of vitamin B_1, this change accompanying the extra oxygen uptake. Much of the evidence presented up to this point is consistent with the idea that the vitamin acts directly upon the pyruvate. We hesitate to take this at its face value. In spite of the constancy of oxygen uptake, there is not much agreement between the O_2/P.A. values. Though the errors involved in the estimation of small differences admittedly reach a high value in Table V, the variations appear to be outside the limits of error. It must be remembered that in the earlier experiments, the vitamin effect failed with pyruvate in tissue which reacted in presence of lactate. Further we have found in a few experiments at p_H 6·6 that pyruvate functions in place of pyro-

phosphate in inducing good oxygen uptake with vitamin, but the disappearance of pyruvate and hence the ratio is again variable (Table VI). (*Cf.* Peters and Sinclair [1933] for behaviour at p_H 6·6.)

Table VI. *Respiration in pyruvic acid at* p_H *6·6 (estimated as B.B.S.).*

	P.					P. + V.			
Exp.	Initial* pyruvate mg.	Weight* tissue g.	Pyruvate* (2 hours) mg.	Pyruvate disappearing mg. per g.	O_2 uptake μl. per g.	Weight* tissue g.	Pyruvate* (2 hours) mg.	Pyruvate disappearing mg. per g.	O_2 uptake μl. per g.
563 (*b*)	[9·81]	0·2454	8·7	[4·6]	1060	0·1991	8·5	[6·5]	1820
570 (*a*)	11·95	0·2194	10·5	6·6	1410	0·1949	9·9	10·5	2425
572 (*a*)	11·5	0·2633	10·4	4·2	1270	0·2519	9·7	7·15	1800
(*b*)	11·5	0·1785	10·4	6·15	1215	0·1972	10·1	7·1	1715
575 (*a*)†	[5·1]	0·1866	4·35	[4·6]	1220	0·1866	4·15	[5·0]	1505
(*b*)†	—	0·1858	4·15	[4·6]	1165	0·2001	3·96	[5·8]	1600

Summary.

Exp.	Pyruvate mg./g.	O_2 μl./g.	O_2/P.A.
563 (*b*)	−1·9	+ 760	400
570 (*a*)	−3·9	+1015	260
572 (*a*)	−3·0	+ 530	180
(*b*)	−1·0	+ 500	500
575 (*a*)†	−0·4	+ 285	700
(*b*)†	−1·2	+ 435	360

* Expressed per sum of duplicate bottles.
† Lactate also present.
(*a*) Without pyrophosphate.
(*b*) With pyrophosphate.

The balance of the evidence inclines to the view that pyruvate disappearance is really consequent upon some other change, and that the vitamin catalyses some other reaction. The sum total of these reactions indicates that about two molecules O_2 are taken up for one molecule of pyruvic acid disappearing; the average ratio for the lactate experiments is 660 μl./mg., for pyruvate 450 μl./mg.; calculated for quotient 2 mol. O_2/1 mol. P.A. = 506.

According to Sinclair [1933] the R.Q. for the extra oxygen uptake due to vitamin in lactate-phosphate solution is 1·6. Hence the average for lactate (660 = 2·5 mol. O_2 approx.) should give 3·75 mol. CO_2 per 1 mol. pyruvic acid disappearing.

For complete oxidation we should have

$$CH_3.CO.COOH + 5O = 3CO_2 + 2H_2O,$$

and R.Q. = 1·2. There is therefore evidence that more CO_2 is being evolved than is required for complete combustion of the pyruvic acid, which indicates entry of some other substance into the oxidation system.

We have the curious paradox that at p_H 7·3 pyrophosphate seems to be needed for pyruvate extra respiration, whereas at p_H 6·6 pyrophosphate is required for lactate extra respiration; at this p_H however pyruvate will function in place of pyrophosphate in restoring catatorulin effect in the presence of lactate. In this complex effect, there appears to be no co-operation of α-glycerophosphate [Peters and Sinclair, 1933], which might have been expected upon the Meyerhof scheme. It is possible that both pyruvate and pyrophosphate are acting as H acceptors at p_H 6·6.

The substance formed from pyruvate in the avitaminous brain is not yet known. Since Meiklejohn [1933] showed that there was no change in lactate accompanying the extra O_2 uptake, and since there is some variation in the ratio O_2/P.A., it seems to be unlikely that lactate is the main substance. In any case, such a conversion involves a reduction.

The simplest explanation of the accumulation of pyruvic acid in the avitaminous tissue and its disappearance in presence of vitamin B_1 is that pyruvic acid is a normal intermediary stage in some phase of carbohydrate metabolism. This is in essence the Embden-Meyerhof view. The phenomena embodied in the new anaerobic fermentation schemes evidently take some part in aerobic respiration too; according to Meyerhof and Kiessling [1933] fluoride should interfere with the appearance of pyruvic acid from phosphoglycerate, and iodoacetate with its disappearance. Consistently with this, we find that no increased pyruvate appears in normal brain tissue respiring in lactate, though a large increase occurs in the presence of iodoacetate (see Exps. 541 and 592, Tables I and VII). Fluoride (Exps. 535, 580, Table I) much reduces the pyruvate formed

Table VII.

Normal brain.

Respiration	Substrate	(Hours) 0–½	½–1	1–1½	1½–2	Total (2 hours)
(μl./g.)	L.	2660	2360	1900	1430	4170
	L. + iodo.	2460	1580	870	340	2625
	L. + fluoride	1470	1310	715	520	2005

Pyruvate estimations.

Substrate	B.B.S.	2:4-dinitro.	%	Tissue weight	– P.A./g.	O_2
L.	0·315	0·255	81	0·170	1·5	4170
L. + iodoacetate	0·665	0·512	77	0·185	2·77	2625
L. + fluoride	0·132	—	—	0·0792	1·33	2005

L. – L. + iodoacetate. Ratio O_2/P.A. = 825.

by avitaminous brain in lactate solutions. Both poisons reduce the O_2 uptake. Other known origins for pyruvate can probably be dismissed with exception of oxaloacetic acid [Krebs, 1933]. Origin from alanine seems mainly confined to kidney [Krebs, 1932], and that from lactic acid [Banga *et al.* 1932; Barron and Hastings, 1933] is not blocked by fluoride (personal communication from Dr E. Boyland). The appearance of pyruvate during respiration of brain tissue *in vitro* and its inhibition by fluoride provide evidence of the re-entry of lactate into some fermentation system; consideration of this must be postponed until we have more precise data of the action of these poisons upon the catatorulin effect.

Note. The figures throughout are given in terms of tissue wet weight, and not as Q_{O_2}, because there seems to be no advantage in the use of this symbol in this comparative work. The values can be readily translated by considering the dry weight to be 20 % of the wet weight values. $Q_{O_2} = 10 \equiv 2000$ ml./g.

SUMMARY.

1. Disappearance of pyruvic acid and of bisulphite-binding substances accompanies the extra oxygen uptake induced by the action of crystalline vitamin B_1 in pigeon's brain tissue respiring *in vitro*. Estimations have been made by the method of Clift and Cook and by a modified Case method.

2. Pyruvate also disappears when substituted for lactate.

3. The average value for the quotient extra O_2 uptake/pyruvic acid disappearing approximates to 506, representing 2 mols. O_2 taken up to 1 mol. pyruvic acid disappearing, but there are wide variations, which lead to the belief that disappearance of pyruvate is an indirect effect of vitamin action.

4. The phenomena are consistent with the view that pyruvic acid is a normal intermediary in the metabolism of pigeon's brain tissue. In agreement with the Embden-Meyerhof fermentation scheme, it accumulates with respiring normal brain tissue in presence of iodoacetate and not of fluoride.

We are indebted to Mr H. W. Kinnersley for a supply of the vitamin solutions, to R. W. Wakelin for skilful help and to the Medical Research Council for grants in aid of the research.

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Note added in proof by R. A. P. In a recent paper [Birch and Mann (1934), *Biochem. J.* **28**, 622] it is stated that we assumed that the oxidation affected was that of lactic acid. Reference to the papers in question, particularly Gavrilescu *et al.* [1932, p. 445], will convince that we were pedantically careful to write oxygen uptake in presence of lactate. In regard to the later criticism of the conclusions drawn from the previous pyruvate experiments, these authors seem to have overlooked the important point that some of the experiments then performed showed only trifling increases with pyruvate and vitamin as compared with pyruvate alone. Later in their paper, they refer to our original statement that 1/10 pigeon dose produced a maximum increase. Unfortunately this has not been corrected in print, though a correction can be inferred from reference to Passmore *et al.* [1933], where about 1/2 pigeon dose produced a nearly maximum effect. The crude preparation originally used was found upon subsequent test to contain more vitamin than had been supposed.

The melting-points in the above paper are corrected. There is some doubt in the literature as to the exact melting point of pyruvic acid 2:4-dinitrohydrazone. The most usual figure obtained is 216°. We have, however, had in our hands a specimen which melted at 218°. It is hoped that sufficient of the compound from the lactate experiments may become available for further confirmation by analysis.

CXXXII. VITAMIN B_3.

By JOHN RICHARD O'BRIEN.

From the Department of Biochemistry, Oxford.

(*Received May 2nd, 1934.*)

Of the components of the vitamin B complex, three have been considered necessary for the nutrition of the pigeon: vitamins B_1, B_3 and B_5. Williams and Waterman [1928] reported that pigeons on a diet deficient in the vitamin B complex required for weight recovery a water-soluble, heat-labile factor. This factor differed from the antineuritic vitamin B_1 and the alkali-stable rat factor B_2. Their belief in the existence of this factor was based on the observation that the addition of a special activated fullers' earth preparation of vitamin B_1 cured polyneuritis and checked the decline in weight of birds on deficient diets but failed to restore weight even when large doses were administered. Autoclaved yeast given as a further supplement had no effect, whilst dried yeast produced a gain in weight. Substitution of the synthetic diet for whole wheat also caused a similar increase. The factor postulated to explain these results was designated vitamin B_3.

Confirmation of the views of Williams and Waterman came from several sources. Randoin and Lecoq [1926] had previously stated that pigeons required in addition to vitamin B_1 a growth-promoting factor which was destroyed by autoclaving in an alkaline medium. In discussing vitamin B_3, Lecoq [1931] expressed the opinion that it was identical with the nutritional factor of Randoin and Lecoq. Carter *et al.* [1930] produced evidence which suggested that the pigeon needed, besides vitamin B_1 and B_3, a third alkali-stable factor, vitamin B_5. At the same time, they supported the claims of Williams and Waterman by showing that amounts of vitamin B_1 as large as 20 pigeon day doses caused no gain in weight in birds on polished rice. The possible existence of some such factor as that postulated by these workers needs consideration since it offers an explanation of the fact that quite large doses of concentrates of vitamin B_1 are required together with vitamin B_3 to cause weight increase. Further, Eddy *et al.* [1930] thought that the factor B_3 was essential for the normal growth of the chick as well as the pigeon. They also demonstrated the extreme lability of vitamin B_3 to heat and alkali.

Williams and Eddy [1931] jointly stated that vitamin B_3 was nothing more than an abundant supply of vitamin B_1, ten to twenty times the antineuritic dose. Nevertheless, they could not be certain that their purest preparation of vitamin B_1 was free from B_3. Their opinion was supported by the work of Morris [1933]. She showed that the properties of vitamin B_3 were very similar to those of vitamin B_1 and that pigeons on a diet of autoclaved whole wheat gained in weight when given large doses of vitamin B_1.

In view of the statement of Williams and Eddy, it was considered desirable to report such evidence as we had obtained in an attempt to elucidate the problem of vitamin B_3. The results suggest that pigeons utilise in addition to the vitamin B_1 a factor for weight restoration. We found that supplements of large

doses of vitamin B_1 concentrate given to birds on a polished rice or autoclaved whole wheat diet had no effect upon their weight. Moreover, it was possible to prepare a concentrate of vitamin B_3 which retained its antineuritic curative properties when its weight-restorative potency had been destroyed. Preliminary experiments tend to indicate that the vitamin B_3 effect is not to be attributed to an insufficiency of protein in the basal diet.

Method of assay.

The adult pigeons, used in this investigation, were fed on polished rice, autoclaved at 15 lbs. for 3 hours to destroy completely vitamin B_1. They were weighed every alternate day at 4 p.m. Twice weekly, they were given by mouth 8–10 drops of cod-liver oil as a supply of vitamins A and D. It has been stated that pigeons do not require vitamin C [Sugiura and Benedict, 1923], which is supported by the fact that vitamin C concentrates prepared from lemon juice as a source of vitamin B_3 had little effect. On the diet, the birds dropped to 60–70 % of their maximum weight in a space of 14–21 days which approximates to the length of the storage period of the B_3 factor. To ensure the depletion of the factor, the birds were taken to maximum weight on whole wheat or by additions of 1 g. dried yeast or 3 g. wheat germ daily and then dropped a second time on polished rice. They were now given by mouth 3 doses of vitamin B_1 daily in the form of an alcoholic concentrate prepared according to the method of Kinnersley and Peters. This alcoholic concentrate is considered to contain, together with the antineuritic vitamin B_1, the factor B_5, the rat factor B_4, and possibly also the factor Y of Chick and Copping [1930]. Throughout the present communication, when speaking of vitamin B_1, we refer to the impure alcoholic concentrate. Under these conditions birds can be maintained at surprisingly constant weight far below their maximum for periods as long as 6 months, the fluctuation in weight being never greater than ± 5 g. This weight maintenance has been observed with 60 birds and amply supports the findings of Carter *et al.* [1930]. Under such treatment the birds develop no symptoms peculiar to vitamin B_3 deficiency. The only ill effects seen so far have been occasional cases of birds showing ruffled feathers, lassitude and loss of appetite.

Concentrates and foodstuffs to be tested can now be given into the crop by pipette. Groups of 5 birds were employed to test any preparation. It is to be noted that although three doses of vitamin B_1 suffice for weight maintenance, experiment shows that at least six are necessary in conjunction with a vitamin B_3 preparation to promote weight recovery.

Results.

The richest sources of the factor B_3 proved to be wheat germ and dried yeast. The effect of these supplements on weight is illustrated in Fig. 1 where it is seen that doses of 3 g. wheat germ and 2 g. dried yeast produce a weight recovery practically equal to that obtained on replacing by whole wheat a diet of polished rice supplemented by vitamin B_1 (Fig. 2). Some gain in weight was caused by egg-yolk and to a smaller extent by malt extract and marmite. No effect was seen with egg-white in doses equal to 1 egg per day, liver powder or milk (50 ml./day) even when the dosage of vitamin B_1 was increased to ten times the antineuritic dose.

The restorative effects on weight of the wheat germ and yeast could not be reproduced by supplying the birds with large doses of the concentrate of Kinnersley and Peters. Doses of vitamin B_1 of forty times the curative amount

have had no influence upon weight. Such doses would presumably contain adequate supplies of the other factors present. A concentrate of vitamin B_2 was prepared from aqueous extract of yeast by lead precipitation. The precipitate was decomposed by warming with sulphuric acid, and the last traces of

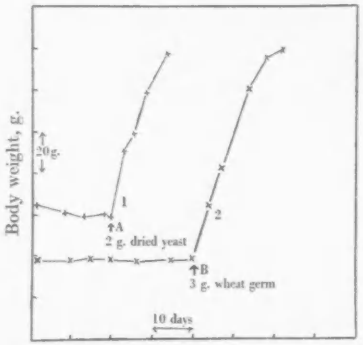

Fig. 1.

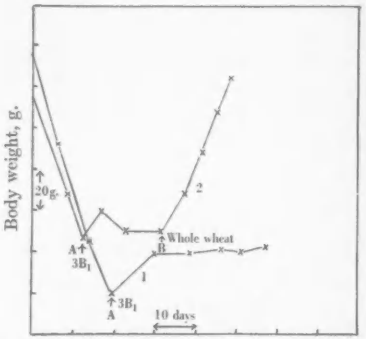

Fig. 2.

Fig. 1. Weight recovery with wheat germ and dried yeast. Typical curves obtained on giving dried yeast (curve 1 A) or wheat germ (curve 2 B) to birds on polished rice + 6 doses vitamin B_1.

Fig. 2. Weight recovery with whole wheat. Curve 1 shows drop in weight of bird on polished rice. At A, 3 doses B_1 are given. Curve 2 shows a similar case. At B, a whole wheat diet is substituted for polished rice + 3 B_1.

lead were removed by H_2S. The solution obtained was inactive as a source of vitamin B_3 in doses four to five times the amount required to cure young rats of dermatitis and to promote growth of 15–20 g./week. From these experiments, it was concluded that none of the factors investigated was responsible for the vitamin B_3 effect.

Preparation of vitamin B_3 extracts.

Attempts were made to prepare extracts of vitamin B_3 from foodstuffs normal to pigeons' diets—maize, corn, wheat. Aqueous and alcoholic extracts were inactive as supplements. Similar extracts of yeast were likewise without activity. It was found that yeast extracted with 97 % alcohol at 40° retained only 50 % of the original vitamin B_3 activity. Slight activity was shown by extracts of grains prepared by means of acidic solvents. These observations led to the conclusion that vitamin B_3 was being held in some form of chemical combination insoluble in the usual solvents. More drastic chemical treatment was therefore attempted to liberate the factor, the most successful being acid hydrolysis. Potent extracts were obtained by hydrolysis of wheat germ and dried yeast.

The wheat germ extract was prepared in the following manner. Wheat germ was extracted three times at 60° with 97 % alcohol and dried in air. Batches of 75 g. were hydrolysed with 300 ml. N H_2SO_4 by vigorous boiling under reflux for 6 hours. The mixture was filtered while hot and cooled. The main bulk of the sulphuric acid was removed by the addition of baryta or barium carbonate until the p_H was about 2. Before dosing, these concentrates were brought with NaOH to p_H 6·8. Given in doses equivalent to 3 g. of wheat germ, the hydrolysate caused an immediate increase in weight in pigeons (Fig. 3).

The properties of the extracts are as follows: There is present little or no protein matter, since the usual protein reagents, trichloroacetic acid, alcohol, sodium tungstate, ammonium sulphate and sodium sulphate caused negligible precipitation under varied conditions. Amino-acids are present together with some reducing substances. The extracts contain vitamin B_1. Tests on two groups of 5 birds in head retraction showed that on an average, there are 4–5 doses of B_1 in one dose of hydrolysed wheat germ. The peculiar property of the extracts is the rapid loss in vitamin B_3 activity on standing at room temperature or in cold store for 6–8 days. In Fig. 3, it is seen that a rapid rise in weight occurs with a freshly prepared hydrolysate followed by decline or maintenance in weight. The addition of another fresh extract renews the weight increase.

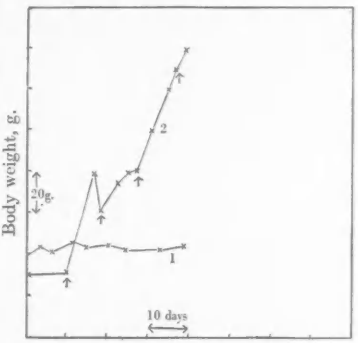

Fig. 3. Weight recovery with hydrolysed wheat germ. Curve 1, control bird on polished rice + 6 doses B_1. Curve 2, bird receiving in addition dose of hydrolysed wheat germ ≡ 3 g. wheat germ daily. The arrows indicate fresh preparations of hydrolysate.

It seemed probable that the inactivation of vitamin B_3 arose from oxidation. Such a view was supported by the fact that when air was drawn through the hydrolysed wheat germ preparations at p_H 2 for 1 hour almost complete loss of activity was observed. Similarly, after treatment with hydrogen peroxide under the same conditions, the extracts produced no effect on weight. Further, the loss in activity can, to some extent, be inhibited by bubbling nitrogen through the mixture during hydrolysis and storing the preparations in nitrogen or *in vacuo*. By such procedure, the activity can be preserved for 10–12 days. Other attempts at preservation with carbon dioxide atmospheres, however, caused inactivation. Little success was obtained with the addition of reducing substances such as sulphite as preservatives. The vitamin B_1 content is unimpaired by oxidation with air; tests on a group of 5 polyneuritic birds revealed that the original 4–5 doses of vitamin B_1 per dose of hydrolysate were retained.

Attempts at fractionation of vitamin B_3 preparations.

The very labile character of vitamin B_3 has rendered difficult attempts to prepare more potent extracts. As mentioned above, protein precipitants are useless. Fractionation with phosphotungstic acid and mercury salts have also proved unsatisfactory. With lead acetate, on some occasions, at least 50 % of the activity has been precipitated but the results have been so variable that it was impossible to use this reagent. It can be concluded from these experiments that the factor B_3 behaves quite differently from vitamin B_1.

Behaviour of vitamin B_3 towards heat.

Eddy *et al.* [1930] considered that vitamin B_3 was extremely heat-labile, being destroyed at temperatures above 60°. On the other hand, Carter *et al.* [1930] state that the vitamin B_3 potency of whole wheat is not completely destroyed after 5 hours' steaming. The conclusions reached by these two groups of workers are directly contradictory. From our experiments, in its behaviour

towards heat the factor B_3 is somewhat similar to vitamin B_1 in that it is more susceptible to moist than to dry heat. We have shown that vitamin B_3 resisted boiling with N H_2SO_4 for 6 hours, which indicates a stability greater than would be expected from the results of the American workers. We have also observed that complete destruction of the factor occurred on autoclaving whole wheat, wheat germ and yeast at 120° for 3 hours while heating these materials at 120–160° for 2 hours hardly impaired the activity. The divergent results of different investigators suggest that the stability of vitamin B_3 is probably dependent upon its state of combination in the foodstuff.

The relation of protein to the vitamin B_3 effect.

Considerable criticism has been levelled at the postulation of factors other than vitamin B_1 being essential for maintenance nutrition of pigeons, on the basis that the experimental diets were not always adequate in respect to other nutrients. The use of polished rice as a basal diet has been particularly condemned. The question of an insufficiency of mineral salts and other vitamins does not arise in our experiments. Further, the caloric requirements are satisfied. Food intakes were measured, and it was found that a pigeon on a polished rice diet receiving 6 doses daily of vitamin B_1 ingested 20 g. daily, the caloric value of which is 71. On a whole wheat diet, which supplies all the essentials for pigeon nutrition, a bird will consume 25 ± 5 g. daily; caloric value 86 ± 17. Sugiura and Benedict [1923] found that pigeons could be maintained on 20 g. of a synthetic diet of a caloric value of 70. The discrepancies in the caloric values of the amounts of different diets consumed daily are hardly sufficient to explain the failure of vitamin B_1 concentrates to restore weight in pigeons on polished rice.

Since the low protein content of polished rice might be responsible for the failure, experiments were made in which protein was given to birds. 2 g. of caseinogen were given daily to birds on rice supplemented by 6 doses of vitamin B_1. No response was observed. When, however, the dosage of vitamin B_1 was doubled, rapid increase in weight was seen in the first 4–7 days of the experiment followed by large fluctuations in the next 7 days. Two groups of 5 birds were used in the experiments and in no case was a rise to maximum weight obtained. In another series of experiments, 2 g. caseinogen were given to birds receiving hydrolysed wheat germ equivalent to 3 g. wheat germ daily in addition to the vitamin B_1 supplement. Complete recovery to maximum weight was obtained (Fig. 4). In Fig. 4, it is shown that the weight increase ceased when the vitamin B_3 concentrate lost its activity.

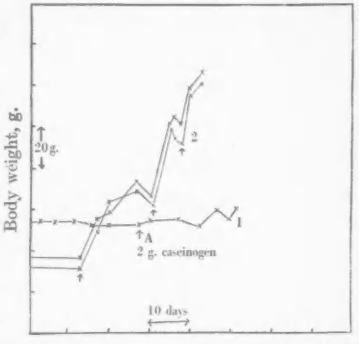

Fig. 4. Caseinogen supplements. Curve 1, control bird on polished rice + 6 doses B_1 daily. At A, 2 g. caseinogen given daily. Curve 2, birds on polished rice + 6 doses B_1 + hydrolysed wheat germ $\equiv$ 3 g. wheat germ + 2 g. caseinogen daily. The arrows indicate fresh preparations of hydrolysate given when decrease in weight occurred with loss in activity of vitamin B_3.

Morris [1933] has tried to overcome the difficulty of the insufficiency of protein by the use of autoclaved whole wheat as a basal diet. She obtained recovery in weight by giving doses of vitamin B_1 roughly six to twelve times the dose curative of polyneuritis, and concluded that

the vitamin B_3 effect was probably a function of a large quantity of vitamin B_1. Our experience differed from that of Morris. A group of 10 birds was placed on whole wheat autoclaved at 120° for 3 hours, and when they had dropped to 60–70 % of their maximum weight, they were given 6 of doses vitamin B_1 daily. After slight fluctuations, the weights of the birds became constant. No recovery in weight was observed on increasing the amount of vitamin B_1 to 30 pigeon day doses. The disagreement of the results of our experiments on caseinogen supplements with those of Carter [1934] and the failure to confirm Morris on autoclaved wheat are possibly connected with fuller depletion of factors in the preparation of the birds used here, but the matter requires further investigation.

Discussion.

From the experiments so far made, it appears that some factor is required for weight restoration by a pigeon on a diet of polished rice supplemented by an alcoholic concentrate of vitamin B_1. The nature of this factor is doubtful. Our results certainly fail to substantiate the statement of Williams and Eddy [1931] and of Morris that vitamin B_3 is merely an abundance of vitamin B_1, many times the dose curative of polyneuritis. The fact that pigeons rise rapidly in weight when removed from polished rice to whole wheat is difficult to reconcile with this view. The daily intake of a bird on wheat would scarcely provide it with such large doses of vitamin B_1. Hydrolysed wheat germ contains a factor which can be distinguished from vitamin B_1 by its instability to oxidation. The mode of preparation of the extract together with its properties suggest that the factor may be an amino-acid. It may, in fact, be one or more of the known amino-acids which, owing to the nature of the extract, break down readily on oxidation. But no evidence has yet been obtained by us in experiments in which known amino-acids such as histidine (20 mg. given daily to 2 birds), tryptophan (20 mg. daily to 2 birds), or cystine (50 mg. daily to 3 birds), present in negligible amounts in polished rice, have been administered together with vitamin B_1 that such a view is correct. In fact, the experiments with caseinogen supplements suggest that the vitamin B_3 effect does not arise from a faulty basal diet.

Summary.

1. Supplements of 20–30 doses of vitamin B_1 fail to restore the weight of pigeons on a polished rice or autoclaved whole wheat diet.

2. Extracts can be prepared from wheat germ which restore the weight of pigeons on polished rice supplemented by vitamin B_1. These extracts are inactivated by oxidation.

3. Birds on polished rice + 6–12 doses daily of vitamin B_1 fail to recover weight when given 2 g. caseinogen daily.

4. It is concluded that the pigeon requires in addition to vitamin B_1 a factor to restore weight.

I wish to express my thanks to Prof. Peters for his advice and criticism throughout this work and to the Department of Scientific and Industrial Research and the Medical Research Council for grants.

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CXXXIII. MAINTENANCE NUTRITION IN THE PIGEON AND ITS RELATION TO HEART BLOCK.

CYRIL WILLIAM CARTER.

From the Department of Biochemistry, Oxford.

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In a previous paper [Carter, 1930] it was shown that pigeon heart block which develops on a diet of polished rice could be cured if the birds received daily rations of whole wheat constituting not less than about 60 % of the total food intake. Vitamins B_1 and B_2 and the fat-soluble group of vitamins were found to be ineffective in restoring normal heart function when administered as supplements to the diet of polished rice. Heart block in the pigeon is therefore a condition of dietary origin. The question remains whether it is due to the deficiency of a specific factor as is the case in polyneuritis, or whether it arises from a deficiency of one or more of those factors which contribute to maintenance nutrition at maximum weight.

In some experiments to be described it will be shown that pigeons previously depleted on a diet of polished rice can, in a majority of cases, be restored to maximum weight by appropriate supplements to this diet without inducing any recovery of normal heart function. Substitution of an extract of hydrolysed wheat germ for the supplements results in a complete recovery from heart block without further significant change in weight. It will be further shown that birds previously brought to maximum weight on whole wheat can be maintained at this level on a diet which fails to protect them from the onset of heart block. The fact, previously recorded, that birds depleted on polished rice, when fed on whole wheat generally recover normal heart rhythm at a time when restoration of body weight is far from complete, has been confirmed. Finally the protein intake of birds on a polished rice diet has been examined, and its significance in relation to the problem of pigeon nutrition is considered.

Method.

Birds under test were kept singly in small cages in order to obtain measurements of the food intake. Observations on body weight were made at the same time each morning in order, as far as possible, to avoid fluctuations due to variations in the amount of food present in the crop. Heart rate and rhythm were noted at frequent intervals.

The source of vitamin B_1 employed was a yeast concentrate prepared according to the method of Kinnersley and Peters [1927] by the extraction of activated norite charcoal with 50 % acid alcohol[1]. In all cases cod-liver oil has been given at regular intervals. Caseinogen (Glaxo, "Vitamin B-free") was

[1] This partially purified concentrate may contain, in addition to vitamin B_1, another factor, vitamin B_5, which has been suggested as being necessary for the pigeon [Carter *et al.*, 1930].

extracted continuously in a Soxhlet apparatus with 90 % alcohol for 48 hours and dried. It has been assumed in this paper that caseinogen treated in this manner provides a source of protein free from possible contaminating substances which might influence the nutrition of the pigeon. Before administration by pipette portions of 2 g. were weighed out, suspended in 20 ml. Ringer solution and brought to p_H 7·0 by addition of caustic soda. Hydrolysed wheat germ was prepared by heating for 3 hours with *N* hydrochloric acid and filtering hot on a Büchner funnel. This method, which differs from that employed by O'Brien [1934], was used daily to prepare the extract in order to avoid the possibility of its deterioration on standing in air.

Experimental.

The earlier experience of the effect of marmite, which in daily doses of 1·5 g. administered over periods of 30 days and upwards showed curative activity in regard to heart block in a certain proportion of cases, prompted a hope that the factor concerned might be fractionated from yeast. The negative results which have invariably been obtained with vitamin B_1 concentrate suggested that preparations from the precipitate which appears on treatment of an aqueous yeast extract with basic lead acetate might be effective. The results have unfortunately been variable and do not at present justify detailed consideration.

The lead precipitate, after treatment with sulphuric acid followed by hydrogen sulphide to remove all traces of lead, when administered to pigeons fed on polished rice is entirely without influence either in preventing decline in weight or in improving the heart condition. In an experiment in which this extract was supplemented by daily doses of an antineuritic concentrate together with 2 g. caseinogen 2 birds showed restoration of normal heart function within 20 days, while in another bird definite improvement though incomplete cure was noted. In all three cases there was gradual recovery over a period of 50 days to maximum weight. Repetition of this experiment with similar treatment resulted in complete cure of heart block in one bird, partial recovery in a second, and no improvement in a third. Restoration to weight maximum was observed in two of these birds while in the third the rise of weight, though substantial, failed to reach that obtained on whole wheat.

These preliminary experiments made it important to ascertain whether the induction and cure of heart block could be dissociated from factors associated with maintenance or restoration of normal body weight.

Effect of yeast concentrate and caseinogen on pigeon nutrition.

A group of 8 birds was placed on a diet of polished rice for periods varying from 12 to 24 days. During this time the usual fall of weight and onset of heart block occurred. In one case acute polyneuritis developed before treatment was commenced. There is at present insufficient evidence to indicate whether variation in the duration of the depletion period has any influence on the subsequent response. A control group of 3 birds was given daily doses of a vitamin B_1 concentrate in amounts equivalent to 12 units daily.

Table I and Fig. 1 show that the progressive decline in weight is arrested and that there is some rise which is less marked than that observed with birds receiving in addition a daily supplement of 2 g. caseinogen. This confirms previous experiments in which maximum weight has not been fully restored by supplements of yeast concentrate or marmite to the basal diet of polished rice.

Table I. *Effect of polished rice and 12 units vitamin B_1.*

Bird	Maximum weight g.	Weight after depletion on polished rice	Duration of depletion days	Final weight	Duration of curative period days
880	396	287	17	338	28
110	400	250	24	260	28
111	360	218	24	255	28

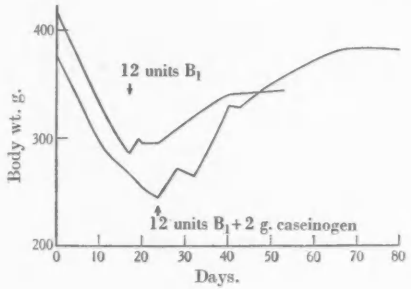

Fig. 1.

Table II records similar data for those birds on polished rice receiving daily 12 units of vitamin B_1 and 2 g. caseinogen.

Table II. *Effect of polished rice supplemented by 12 units vitamin B_1 and 2 g. caseinogen.*

Bird	Maximum weight g.	Weight after depletion on polished rice	Duration of depletion days	Final weight	Duration of curative period days
401	324	268	12	325	59
357	376	247	24	380	53
722	468	286	24	398	57
622	392	268	15	376	54
485	390	285	15	380	54

It will be seen that of these birds only one, No. 722, failed to reach maximum weight in the course of the experiment. In this respect their behaviour is in agreement with that of the birds in the earlier experiments.

In another experiment 2 birds, previously brought to maximum weight on mixed corn, were placed on polished rice supplemented by 2 g. caseinogen and 12 units of vitamin B_1 daily. After an initial fall of weight of 28 and 16 g. respectively during the first 4 days there was almost no change throughout the experimental period which lasted for 37 days (Table III).

Table III.

	Day	Bird 622	Bird 485
Initial maximum weight	0	418	434
Weight after commencement of experimental diet	4	402	406
	10	405	411
	20	399	404
	30	390	396
	37	398	401

The rather unexpected result of these experiments raises a number of points of interest. In the first place the rise of weight to maximum is notably slow in comparison with the rise seen on whole wheat. Of the birds referred to in Table II full weight was not attained in two cases until the 40th day while in another it was delayed until the 59th day. This is about four times as slow as in the case of whole wheat. The fact that the weight rise is so gradual under these conditions raises the possibility that experiments of short duration may lead to misleading conclusions.

While the attainment of maximum weight upon a given diet may, subject to the possibility of storage of essential factors during the initial depletion period, be justifiably held to indicate that such a diet is "adequate" for full nutrition it does not necessarily follow that it contains a satisfactory balance of essential dietary constituents such as is present in whole wheat. In two respects a diet of polished rice may be ill-balanced. In 4 birds it was found that daily administration of 6 units of vitamin B_1 was inadequate with caseinogen, whereas an increase of dosage to 12 units sufficed to promote slow continued rise of weight. This large daily requirement of vitamin B_1 may be more apparent than real since the concentrate in which it is administered is also the source of vitamin B_5, another essential dietary constituent for maintenance in the pigeon, and it is possible that the latter may here be a limiting factor. On the other hand, as suggested by Williams and Eddy [1931], there may be a real difference in the requirement of vitamin B_1 for cure of polyneuritis and for maintenance of full nutrition. Morris [1933] claims that, with a basal diet of autoclaved wheat, the dose of a vitamin B_1 concentrate necessary to effect complete weight recovery after depletion exceeds the dose required for cure of polyneuritis by an amount which varies from six to sixteen times with different types of yeast concentrate. On the other hand where the basal diet is one of polished rice full recovery does not occur even if as much as 30 curative doses of vitamin B_1 are administered daily [Carter *et al.*, 1930]. The difference in the nature of the basal diet is important. The factor which appears to limit weight recovery on polished rice supplemented by a yeast concentrate is a relative deficiency of protein. Moreover a difference in the type of protein available on diets of wheat on the one hand and polished rice supplemented by caseinogen

Table IV. *Average daily intake.*

	Bird 880			Bird 110			Bird 111		
	Total cals.	Protein g.	Days on test	Total cals.	Protein g.	Days on test	Total cals.	Protein g.	Days on test
Wheat	79·23	2·50	9	72·34	2·29	11	80·95	2·56	5
Rice with 5 units vitamin B_1	71·42	1·18	5	—	—	—	—	—	—
Rice with 10 units vitamin B_1	83·20	1·37	10	60·70	1·00	10	59·27	0·98	10
Rice with 12·5 units vitamin B_1	85·34	1·41	11	55·34	0·92	9	71·42	1·18	10

	Bird 622			Bird 401		
	Total cals.	Protein g.	Days on test	Total cals.	Protein g.	Days on test
Wheat	89·57	2·83	6	82·7	2·61	6
Rice with 2 g. caseinogen, and 12 units vitamin B_1	85·0	3·15	52	87·8	3·18	13

on the other may perhaps contribute to the difference in the time required for weight recovery in the two cases. Table IV shows the calory and protein intake for birds on diets of polished rice supplemented by a yeast concentrate, with and without addition of caseinogen.

Birds 110 and 111 show some diminution in their daily calory intake as compared with that on whole wheat, whereas in the case of bird 880 this appears to be satisfactory. Sugiura and Benedict [1923] found, in experiments in which pigeons were forcibly fed on a diet containing 22 % caseinogen, that a daily intake of about 70 calories sufficed for normal growth and maintenance at full weight.

There is however in all birds on a rice diet a substantial diminution of the daily protein intake. On the other hand birds 622 and 401, which rose to their maximum initial weight on a rice diet to which were added 2 g. caseinogen in addition to the yeast concentrate, show daily calory and protein intakes which compare favourably with the intakes on whole wheat.

The biological value of the protein of polished rice has been regarded by Osborne *et al.* [1915] as high, but on the other hand its protein content is only about 50 % of that of whole wheat. There is at present no information as to the minimum protein requirement for the pigeon at maximum weight and the figures for protein intake on a polished rice diet do not therefore prove that this intake is inadequate. However, the fact that an additional protein supplement in the form of caseinogen to the basal diet enables the bird to regain full weight suggests that insufficient polished rice is consumed to meet its protein requirement. In this connection the observations of Aykroyd [1930], on the significance of partial protein deficiency in human beri-beri, may be mentioned.

It is desirable at this stage to define the sense in which the term "maximum weight" has been used in the preceding experiments. It is not here intended to imply that maximum weight represents some fixed and constant level of nutrition for any particular bird, since the body weight at any given moment is determined not only by the nature of the diet which it is receiving but also by its past dietary history and doubtless by other factors. The birds, before experiment, are placed on mixed corn for some weeks, and during this time come into equilibrium at a weight which has been here termed "maximum." If, after a period on an experimental diet having polished rice as its basis, the birds are returned to whole wheat the new weight which is established is not necessarily identical with the former maximum. In some cases, examples of which will be seen in experiments with wheat germ, the new weight level somewhat exceeds the initial "maximum." On the other hand a number of cases have been met with during the course of work in this laboratory in which whole wheat or corn fails to restore the full original weight level. It is not the purpose of this paper to discuss the factors underlying these variations in maximum weight. The facts must be borne in mind in assessing the significance of the experiments with caseinogen which have been described.

Effect of vitamin B_1 and caseinogen on pigeon heart block.

In the experiments described in the preceding section repeated observations were made on the condition of the heart. At the end of the initial depletion period on polished rice irregularity of rhythm and bradycardia, which in some cases was very marked, were observed. For example bird 722, after a depletion period of 24 days, showed a fall of heart rate from 200 per minute to 66 per minute.

It has been frequently observed that some rise of rate is associated with rise of body weight though the persistence of irregularity of rhythm shows

that the underlying disorder of the vagus centres remains. In all the birds whose weights are recorded in Table II heart block persisted through the entire period of the experiment in spite of a weight rise to maximum in four of them. Table V shows records of the heart in two typical birds of this group.

Table V.

Days on experimental diet	Heart rate and rhythm			
	Bird 722		Bird 357	
0	66	Regular	112	Dropped beats
4	144	Dropped beats	144	,,
9	132	,,	164	,,
18	144	,,	148	,,
26	128	,,	164	,,
38	148	,,	156	,,
54	128	,,	160	,,

In the experiment in which birds, having attained maximum weight on a diet of mixed corn, were maintained at this weight on a diet of polished rice supplemented by caseinogen and vitamin B_1 concentrate, heart block developed at the 8th and 15th days respectively and persisted through the experiment lasting 37 days.

These experiments show that normal nutrition as judged by weight tests can be established in conjunction with a dietary deficiency which is revealed by the presence of cardiac arhythmia. Experiments now to be described show that this deficiency can be supplied by a constituent of wheat germ.

Effect of wheat germ on pigeon heart block.

Table VI shows the effect of administration of wheat germ hydrolysed for 3 hours in association with 12 units of vitamin B_1 concentrate.

Table VI.

Bird	Before administration of wheat germ			After administration of wheat germ (6 g.)			Duration of treatment days	Initial maximum weight g.
	Heart rate		Weight g.	Heart rate		Weight g.		
622	140	Dropped beats	376	152	Regular	427	23	392
485	100	,,	380	176	,,	401	16	387
722	160	,,	398	174	,,	423	13	468
357	160	,,	380	192	,,	399	14	376
				Wheat germ 4 g.				
940	124	,,	253	160	Dropped beats	320	39	338
				Wheat germ 8 g.				
				176	Regular	330	4	—
				Wheat germ 4 g.				
921	132	,,	282	160	Regular	313	20	368
				Wheat germ 6 g.				
44	116	,,	299	176	Regular	380	19	362

The first 4 birds had previously received caseinogen and the yeast concentrate with the results already described. Wheat germ extract in doses equivalent to 6 g. cleared up the cardiac irregularity in each case after an interval of 13–23 days. This curative interval is considerably longer than that usually observed with whole wheat, but it should be noted that the method of preparing the extract involves considerable losses, and the actual amount administered

is probably substantially less than the equivalent of 6 g. of dry wheat germ. It will be seen that in three of these birds there is a rise in weight which somewhat exceeds the initial weight maximum. The significance of this fact has already been discussed. The remaining birds received wheat germ extract immediately following the depletion period. Birds 921 and 940 received the equivalent of 4 g. of the extract, and in the case of the latter this dose failed to improve the cardiac condition in a period of 39 days. An increase in the dose to the equivalent of 8 g. resulted in complete recovery of the heart in 4 days.

A final experiment in which a group of birds, after depletion on polished rice, were returned to whole wheat, provides further evidence for the view that restoration of heart block can be effected independently of the state of nutrition of the bird as judged by its body weight. Table VII shows that restoration of the heart occurred within 2–5 days after commencement of a whole wheat diet, and at a time when recovery of body weight was still incomplete.

Table VII.

Bird	Maximum weight g.	Weight after depletion on rice g.	Weight at time of recovery of heart g.	Heart rate after depletion on rice	Heart rate after recovery on wheat	Time for recovery of heart days
142	488	317	400	100 Dropped beats	156 Regular	2
912	346	229	293	128 ,,	240 ,,	5
157	450	342	419	80 Regular	200 ,,	5
23	384	251	310	108 Dropped beats	184 ,,	3

SUMMARY.

The experiments outlined in this paper provide evidence for the view that one factor limiting the restoration, after depletion, of full weight of pigeons on a diet of polished rice supplemented by a yeast concentrate is an inadequate allowance of protein in the diet. Where this partial deficiency is made good by the addition of caseinogen full restoration of weight usually occurs. It is not finally proved that caseinogen, purified in the manner described, provides no factor other than protein, and it would therefore be premature to draw conclusions from these experiments as to the necessity for vitamin B_3 in pigeon nutrition. The evidence does however indicate that maintenance of, or recovery to, full weight may be compatible with the persistence of a cardiac arhythmia of dietary origin. This dietary constituent can be extracted from wheat germ, and its administration in amounts equivalent to 6–8 g. leads to complete restoration of function.

I desire to record my sincere thanks to Prof. Peters for his continued interest and advice.

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CXXXIV. THE COLLOIDAL PHOSPHATE OF MILK.

By GERALD THOMAS PYNE.

From the Department of Dairy Chemistry, University College, Cork.

(*Received April 17th, 1934.*)

The presence of insoluble, or rather colloidal, calcium phosphate in milk has been recognised for a long time; but despite numerous researches both into the type of phosphate present and the nature of its association with other colloids, it cannot yet be said that either of these matters has been definitely cleared up. To judge from the literature, opinion seems to be still divided, firstly, as to whether the phosphate is secondary or tertiary, or a mixture of the two, and secondly, as to whether it is in chemical combination with calcium caseinogenate or merely protected by it. It is hoped in the present paper to present some additional evidence on these points, which appears to favour the view that the phosphate is mainly tertiary and is present in intimate union with the caseinogenate.

Hammarsten [1879] supposed that calcium phosphate is held in solution in milk by calcium caseinogenate, and Lehmann [1894] regarded the two as united to form a double salt in which the phosphate is the tricalcium salt. Lindet's views [1912] appear to be similar. Söldner [1888] concludes that both di- and tri-calcium phosphate are present in milk, the former in somewhat the greater amount, and apparently rejects the hypothesis of a chemical union between these substances and caseinogen. Hoffman and Gortner [1923] and Porcher [1930] also consider the phosphate to be a mixture of the same two salts and their relation to the protein to be solely one of colloidal protection. Van Slyke and Bosworth [1914, 2], while accepting and apparently verifying, by their experiments on the centrifugal separation of milk the merely protective rôle of the caseinogenate to calcium phosphate, consider the latter to be the di-calcium salt exclusively—an opinion apparently shared by Palmer [1924], Grimmer [1926], Wright [1928] and many others. On the other hand, György's experiments [1923] on the pancreatic digestion of milk and of artificially prepared caseinogen-calcium phosphate complexes led him back to the earlier view that some type of definitely chemical combination exists between calcium phosphate and the protein. Recent work by Kometiani [1931] on heated milk, which will be referred to more fully later, also supports this view.

In the course of some work in another connection [1932], the present writer noticed (*a*) that the formaldehyde titration of milk protein was much lower in the presence of colloidal calcium phosphate than in its absence, and (*b*) that the titratable acidity of milk was greatly diminished on addition of a sufficient quantity of a soluble oxalate. The first observation seemed to bear the interpretation, then stated, that the colloidal phosphate and calcium caseinogenate were chemically combined; and the second, which was not indeed new, but was susceptible of a different explanation [*cf.* Pyne and Ryan, 1932] from that given

by Van Slyke and Bosworth [1914, 1], suggested that the phosphate was largely tertiary.

The present paper contains a development of these observations and some others, which appear to confirm these views.

A. *Relation of calcium phosphate to calcium caseinogenate.*

In the previous paper [1932] it was shown that addition of a soluble oxalate (4 % of the saturated potassium salt) to milk increased its formaldehyde titration by about 11 % on the average. For some artificially prepared caseinogen-calcium phosphate complexes in which about one-half of the total calcium was combined with phosphoric acid, the percentage increase in formaldehyde titration was somewhat greater on oxalation, *viz.* about 16 %. (The oxalate is regarded as dissolving the union between the caseinogenate and the phosphate.)

Somewhat similar results have since been obtained with other complexes of the same type, *e.g.* a caseinogen-calcium carbonate, and also with a caseinogen-calcium phosphate complex to which gelatin had been subsequently added. On the other hand, where colloidal calcium phosphate was protected by gelatin, the addition of oxalate did not appreciably affect the formaldehyde titration, and this held even when calcium caseinogenate, subsequently added, was present in the mixture at the time of oxalation and titration. A few typical results are given in Table I.

The caseinogen-carbonate complex was prepared, following Porcher [1930], by bubbling carbon dioxide at ordinary temperature through an alkaline solution of calcium caseinogenate (3·7 % CaO) until decidedly turbid and then removing the excess carbon dioxide by a current of air; the gelatin-calcium phosphate colloid was obtained according to the directions of De Toni [1921].

Table I.

Solution	% total protein	Formaldehyde value ml. *N* alkali per 100 ml. solution	
		With oxalate	Without oxalate
Caseinogen-calcium phosphate complex	2·3	1·30	1·15
,, carbonate complex	2·0	1·14	0·98
Gelatin-calcium phosphate, Complex A	4·5	1·39	1·37
,, ,, B	—	1·32	1·31
,, ,, C	4·75	2·05	2·07
+calcium caseinogenate (2·5 %)			

It is clear that protection of colloidal calcium phosphate (and carbonate) by calcium caseinogenate is quite different in type from protection by other proteins, *e.g.* gelatin. The marked difference is most simply explained by assuming that in the first case, where the properties of the protein are considerably modified by this "protection," the union is more definitely chemical. The potentiometric titration of the complex described later in this paper also favours this view.

In this connection the experiments of Kometiani [1931] on heated milk are of interest. This author attributes some at least of the increased titratable acidity that results from heating to a partial separation of calcium phosphate from its caseinogenate complex, thereby increasing the number of free carboxyl groups in the protein and thus its buffer value and titratable acidity. In apparent confirmation of this view he finds that the formaldehyde titration of milk increases on heating, the increase reaching its highest value of about 15 % on heating milk for half-an-hour at 120°. It is remarkable that a difference of

much the same order is shown (*cf.* Table I) for the formaldehyde titration of calcium caseinogenate according to whether it is associated with calcium phosphate or not.

These findings favour then the views of György, *i.e.* that the relation of calcium phosphate to the caseinogenate is one of chemical union and not merely of colloidal protection. As to the type of union between them, its rapid dissolution under the action of oxalate suggests that the common bond is one or more calcium atoms, and that the complex is thus a double salt of caseinogenate and phosphate. The marked shift in the reaction of the complex towards alkalinity on addition of potassium oxalate, a matter which will be discussed more fully later, makes it probable that the phosphate liberated is largely K_3PO_4 arising from the tertiary calcium salt. The complex should on these views be essentially a double salt of calcium caseinogenate and tricalcium phosphate, and its primary interaction with oxalate might be represented schematically by:

$$(NH_2)_p\text{—}R\text{—}\begin{matrix}(COOH)_l\\(COOCa')_m\\(COO.Ca.PO_4.Ca)_n\end{matrix} + \tfrac{3}{2}n.K_2\overline{Ox} = (NH_2)_p\text{—}R\text{—}\begin{matrix}(COOH)_l\\(COOCa')_m + \tfrac{3}{2}n.Ca\overline{Ox} + n.K_3PO_4\\(COOK)_n\end{matrix}$$

where Ca′ represents for convenience a half-atom of calcium. It is of course not intended to suggest that the phosphate complex should be a definite individual salt, but only a certain type of compound in which however the proportion of phosphate can be varied greatly according to the method of preparation. The evidence in favour of the presence of the elements of tricalcium phosphate in this complex will now be considered more fully.

B. *Nature of the colloidal phosphate.*

Ignoring the small amount of colloidal magnesium phosphate which is present in milk in somewhat the same form, no doubt, as the corresponding calcium phosphate, and regarding the caseinogen-calcium phosphate of milk as essentially a double salt of caseinogenate and phosphate, the exact nature of the latter, whether secondary, tertiary or a mixture of these remains to be determined. In a sense, it may appear immaterial which, for owing to the variation in the calcium content of the caseinogenate with p_H, a compound of, say, dicalcium phosphate and calcium caseinogenate, might be regarded from one point of view as identical with one of tricalcium phosphate and a more acid caseinogenate. For definiteness, however, the colloidal calcium phosphate ingredient of the complex must be considered to be that phosphate which can unite with calcium caseinogenate to form the complex without any considerable change in p_H. This is, in fact, the assumption made by Porcher in his experiments on the synthesis of the complex (see later).

Action of soluble oxalate on milk. The writer and Ryan [1932] have shown elsewhere that addition of a soluble oxalate to milk in sufficient amount (*e.g.* 4 % of a saturated solution of potassium oxalate) reduces the titratable acidity to phenolphthalein by about 1·20 ml. $N/100$ ml. milk. For whey, on similar treatment, the fall in acidity is about 0·30 ml. $N/100$ ml., so that the net alkalinity developed by the interaction of oxalate and the caseinogen-calcium phosphate complex of the milk amounts to about 0·90 ml. $N/100$ ml. The apparent source of most of this alkalinity is K_3PO_4 produced from tricalcium phosphate by the action of oxalate. For solutions of calcium caseinogenate alone develop only very slight alkalinity on addition of oxalate, and any colloidal dicalcium phosphate ($CaHPO_4$) that may be present in the complex will give rise to

K_2HPO_4 which is practically neutral to phenolphthalein[1]. Assuming then that 0·90 ml. *N* represents approximately the amount of acid to convert the tricalcium phosphate of 100 ml. milk *via* K_3PO_4 into K_2HPO_4, the tricalcium phosphate content of milk can be calculated to be about 0·14 g. $Ca_3P_2O_8$/100 ml., containing 0·076 CaO. Söldner [1888] estimates the lime content of the colloidal phosphate to be 0·071 g./100 ml. milk and Porcher and Chevallier [1923] about 0·065 g. The figure obtained from oxalate titration, which represents the lime content of tricalcium phosphate only, does not differ greatly from these values, and the agreement suggests that much or all of the colloidal phosphate of milk is tertiary.

More direct evidence is obtained by comparison of the tricalcium phosphate (calculated from ordinary and oxalate titrations of milk and whey as above) and the total colloidal phosphate for the same sample obtained by analysis. The latter was taken as the difference between the acid-soluble inorganic phosphorus contents of milk and rennet whey determined by the Briggs colorimetric molybdate method and using the technique of Lange and Miethke [1933]. Table II gives some results.

Table II.

Sample	Net oxalate shift ml. *N*/10 per 100 ml. milk	Inorganic P as ml. *N*/10 H_3PO_4 per 100 ml.		
		Total	Soluble	Insoluble
A	8·8	20·0	11·7	8·3
B	8·8	19·9	10·9	9·0
C	12·2	21·7	8·7	13·0

The agreement between the values in the first and last columns is quite close. For strict comparison the former still require to be reduced by about 0·3 ml. *N*/10—approximately the degree of alkalinity to phenolphthalein developed in 100 ml. 3 % calcium caseinogenate on addition of the amount of oxalate here used—a correction which does not materially affect the results. These show that the greater part of the colloidal phosphate, if not all, reacts to oxalate as tricalcium phosphate.

Preliminary experiments on caseinogen-calcium phosphate complexes.

These experiments were made primarily in order to see how far the effects of oxalate on milk could be reproduced on synthetic materials, and they are somewhat qualitative. More detailed examination of the phosphatic complexes is reserved for the next section. It was found that these complexes whether obtained by the action of phosphoric acid on alkaline calcium caseinogenate at about 0° or formed in similar conditions by the successive slow addition of calcium chloride and disodium phosphate solutions to one of nearly neutral caseinogenate (a process which in the absence of caseinogenate gives $CaHPO_4$), showed in all cases, in common with milk, the property of becoming much more alkaline on addition of oxalate, and thus appeared to contain some tricalcium phosphate.

The following results found for complexes prepared by the second method illustrate this point.

[1] The applicability of this oxalate method for the approximate determination of small amounts of tricalcium phosphate, and the correctness of these statements have been checked experimentally on solutions or suspensions of $CaHPO_4$ and $Ca_3(PO_4)_2$, including colloidal suspensions of the latter in gelatin. The last react with oxalate somewhat slowly.

Table III.

	Total P added as ml. $M/10$ H_3PO_4 per 100 ml.	Total oxalate shift of complex ml. $M/10$ per 100 ml.	Oxalate shift of papain filtrate ml. $M/10$ per 100 ml.	Net oxalate shift ml. $M/10$ per 100 ml.
Complex A	11·1	8·2	1·4	6·8
,, B	10·0	7·4	1·5	5·7

An approximate titrimetric estimation of the soluble phosphate in the papain[1] filtrates gave 4·1 ml. $M/10$ and 4·6 ml. $M/10$ per 100 ml. respectively for A and B, so that the $CaHPO_4$ which might have been expected from the method of preparation is apparently replaced in these experiments by almost equal amounts of tricalcium and of soluble phosphates.

No doubt the reaction of the solution changed during the formation of these phosphates, and would need to have been followed to allow more quantitative interpretation of the results, but the conclusion that tricalcium rather than dicalcium phosphate is formed can hardly be avoided. The following experiment offers further confirmation of this view.

A solution of $M/5$ disodium phosphate of p_H 8·47 was added in successive small amounts to a 3 % calcium caseinogenate solution of about the same p_H, *viz.* 8·4, and the hydrogen ion concentration of the mixture was determined after each addition, using the glass electrode. If dicalcium phosphate is formed from the interaction of the two solutions as the inorganic colloid associates with the caseinogenate, the mixture should not differ greatly in p_H from its constituents. If however tricalcium phosphate is formed, from what may be regarded as the elements of $CaHPO_4$, the resulting mixture should become more acid, particularly in the earlier stages. The experimental results, shown diagrammatically in Fig. 1 are in complete accordance with the latter view. To demonstrate that the changes in p_H which occurred on addition of the phosphate to the caseinogenate were not due merely to partial interchange of bases in the latter, *etc.*, a similar series of potentiometric titrations was made on part of the same calcium caseinogenate solution treated with neutral $M/5$ potassium oxalate. These results, shown also in Fig. 1, are entirely different from those with phosphate.

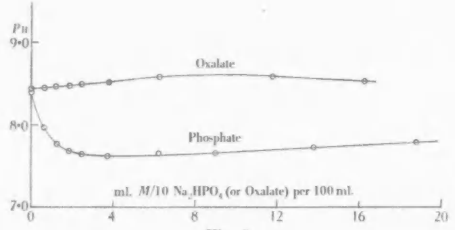

Fig. 1.

Distribution of inorganic phosphorus in caseinogen-calcium phosphate complex.

Porcher and Chevallier [1923] concluded that the colloidal calcium phosphate was the tertiary salt, but Porcher later [1930] formed the opinion, from experiments on the artificial preparation of caseinogen-calcium phosphate complexes,

[1] These solutions contained too little soluble calcium to allow the use of rennet in preparing the serum and papain was employed instead, as by Kometiani [1931].

that the inorganic colloid was neither entirely secondary nor tertiary but a mixture of the two. The complexes were prepared by the addition of sufficient phosphoric acid to decidedly alkaline calcium caseinogenate to bring the whole to about p_H 7·0. On the assumption that the calcium belonging properly to the protein is approximately equal in the complex to that found in pure calcium caseinogenate of the same p_H, he found that 1 g. mol. H_3PO_4 was required in order to neutralise approximately 1·25 g. mol. of excess CaO, a result which corresponded to the formation of di- and tri-calcium phosphates in almost equal proportions. Porcher recognised that a small amount of monocalcium phosphate was formed during the neutralisation, and that dicalcium phosphate is itself slightly soluble, but does not seem to have considered quantitatively the bearing of these facts on the type of colloidal phosphate formed at the same time. It is clear that they may influence the interpretation of the titration results considerably. For, if soluble phosphate is present at a p_H of about 7, it will consist of a mixture of nearly equal amounts of monocalcium and dicalcium phosphates, for which CaO/H_3PO_4 in g. mols. = 0·75. It is only necessary for one-fourth of the total phosphate to be present in this form in order to account for an experimentally found ratio CaO/H_3PO_4 in g. mols. = 1·25, on the basis of the other three-quarters being entirely tertiary.

It was decided therefore to repeat the preparation of these complexes, determining at the same time the proportion of the added phosphate which remained in solution. For this purpose a calcium caseinogenate solution containing about 5 % of the protein and having p_H about 7·0 was prepared by trituration of "Hammarsten casein" with lime water, allowed to stand overnight in an ice-chest and then filtered through hardened paper. To 50 ml. of the filtrate were added 30–40 ml. saturated lime water, and then $M/20$ phosphoric acid was slowly run with constant rapid mechanical stirring into the ice-cooled mixture until it had attained approximately its original p_H of 7·0. After some hours the hydrogen ion concentrations of the phosphate complex and of the original filtered calcium caseinogenate (similarly diluted with water) were determined with the glass electrode. One or two further determinations were also made on the latter solution after the addition of small quantities of lime water, so as to be in a position to allow accurately for the calcium directly associated with caseinogen in the complex, in case the p_H values of complex and original caseinogenate should differ slightly.

The total inorganic calcium and phosphorus contents of the complex were, of course, known from the amounts of lime water and phosphoric acid used in its preparation, but the determination of the soluble phosphate required the removal of the caseinogen-calcium phosphate complex from solution without disturbing the equilibrium of the soluble constituents. Rennet was unsuitable for this purpose as the soluble calcium content was too low and could not be increased, *e.g.* by addition of a soluble calcium salt, without danger of upsetting the equilibrium of the system. The enzyme papain, used successfully by Kometiani [1931] for preparing the serum of heated milk, proved unsatisfactory in the present instance, since the filtrate obtained could not be rendered sufficiently free from nitrogenous matter by trichloroacetic acid and gave cloudy solutions with the Briggs molybdate reagent. Ultrafiltration was finally adopted for the preparation of the serum and the phosphate determined colorimetrically as already mentioned. The calcium associated with phosphate in solution was calculated from the latter for the particular p_H of the complex, using Sørensen's value for the phosphate buffers [*cf. e.g.* Clark, 1928]. The results are shown in Table IV. The figures in the last column entitled "corrected oxalate shift"

Table IV.

% protein	p_H	Inorganic P as ml. $M/10$ H_3PO_4 per 100 ml. Total	Soluble	Insoluble	Inorganic Ca as ml. $M/10$ $Ca(OH)_2$ per 100 ml. Total	Soluble (calc.)	Insoluble	Ratio ml. $M/10$ $Ca(OH)_2$ / ml. $M/10$ H_3PO_4 Total	Insoluble	Corrected oxalate shift ml. $N/10$ per 100 ml.
2·37	6·97	7·40	—	—	9·15	—	—	1·24	—	5·0
2·05	6·91	5·90	1·48	4·42	7·29	1·15	6·14	1·24	1·39	4·0
2·11	6·87	6·60	1·28	5·32	8·51	0·99	7·52	1·28	1·41	4·5

represent the alkalinity developed in the complex on addition of the usual amount of oxalate, less an allowance of 0·5 ml. N to cover the sum of the alkalinities developed in 2 % calcium caseinogenate and the ultrafiltrate from the complex on similar treatment.

The ratio of total inorganic calcium to phosphorus is seen to agree closely with that found by Porcher, but, as expected, allowance for that fraction of these elements present in solution raises the ratio of the remainder which is available for the formation of colloidal phosphate to about 1·40. As the corresponding ratios for dicalcium and tricalcium phosphates are 1·0 and 1·5 respectively, it is clear that this result requires the bulk of the colloidal phosphate to be tertiary but does not exclude however the possibility that some dicalcium phosphate is also present. The values for the shift in reaction of the complex towards alkalinity on addition of oxalate, shown in the last column, are seen to be slightly lower than those for insoluble or colloidal phosphate and thus also support this possibility.

In spite of the suggestion from these results that the colloidal phosphates may be partly secondary, there are other considerations which favour the simpler view that the colloid is entirely tertiary and that the slight divergencies in these experimental results from what might have been expected in such circumstances are due to interfering causes. For instance, it has been assumed that the caseinogen-calcium phosphate complex and the corresponding caseinogenate free from colloidal phosphate will possess the same p_H values in solution. While some such assumption is necessary to prevent the problem becoming indeterminate, it is probably not correct to expect that the two corresponding substances will really have identical p_H values, although they will probably not differ greatly in this respect. Differences in the degree of hydrolysis, *etc.*, which occur, for instance, between the alkali and alkaline earth caseinogenates, are likely to operate here also. Thus, results which make a caseinogen-calcium phosphate complex appear to consist, somewhat improbably, of calcium caseinogenate associated with a mixture of phosphates of which some 80 % is tertiary and 20 % secondary may more properly be susceptible of the simpler interpretation that the complex consists of caseinogenate and tricalcium phosphate only, the union of which is accompanied by a slight change in reaction. Evidence on the point was sought by means of potentiometric titration.

Potentiometric titration of calcium caseinogenate and caseinogen-calcium phosphate complex.

On the assumption that the amount of calcium associated directly with caseinogen in the complex is identical with that present in calcium caseinogenate of the same p_H, the titration curves of the two substances (freed of course from soluble phosphate) given with calcium hydroxide will be exactly similar. If

however this assumption is not strictly correct, as is suggested, the titration curves should differ somewhat. Table V gives the results of some experiments, one of which is also shown graphically in Fig. 2. Solutions of calcium caseinogenate and of various phosphatic complexes prepared from them were made and matured, as previously described, and then dialysed side by side in cellophane sausage skin dialysers for (mostly) 18 hours to remove soluble phosphate from the complex. A little mustard oil was used as a preservative. The solutions which contained some 2–3 % protein were then titrated potentiometrically with lime water, using the glass electrode and allowing an interval of 10 minutes for attainment of equilibrium between each addition of alkali and the succeeding measurement.

Table V.

Exp. 1

Dialysis (18 hrs.)			
Complex		Caseinogenate	
ml. $N/10$ $Ca(OH)_2$ per g.	p_H	ml. $N/10$ $Ca(OH)_2$ per g.	p_H
0·0	6·94	0·0	6·85
1·10	7·82	1·12	7·68
2·20	8·73	2·24	8·73
3·30	9·34	3·36	9·26
4·40	9·71	4·47	9·57

Exp. 2

Dialysis (18 hrs.)			
Complex		Caseinogenate	
ml. $N/10$ $Ca(OH)_2$ per g.	p_H	ml. $N/10$ $Ca(OH)_2$ per g.	p_H
0·0	7·10	0·0	6·82
0·78	7·71	0·80	7·63
1·56	8·54	1·60	8·66
2·34	9·17	2·41	9·11
3·12	9·45	3·21	9·36

Exp. 3

Dialysis (18 hrs.)			
Complex		Caseinogenate	
ml. $N/10$ $Ca(OH)_2$ per g.	p_H	ml. $N/10$ $Ca(OH)_2$ per g.	p_H
0·0	7·10	0·0	6·79
0·69	7·55	0·68	7·41
1·38	8·13	1·36	8·27
2·07	8·76	2·04	8·88
2·76	9·30	2·72	9·16
4·14	9·71	4·08	9·58

Dialysis (66 hrs.)			
Complex		Caseinogenate	
ml. $N/10$ $Ca(OH)_2$ per g.	p_H	ml. $N/10$ $Ca(OH)_2$ per g.	p_H
0·0	7·19	0·0	6·79
0·73	7·82	0·72	7·28
1·46	8·67	1·44	8·11
2·18	9·24	2·17	8·69
2·91	9·48	2·89	9·09
4·36	9·90	4·33	9·57

All the results show that the two titration curves are not identical in shape, the buffer value of the complex being somewhat greater than that of the caseinogenate in the earlier stages of the titration, *e.g.* from about p_H 7·0 to p_H 8·5, and lower thereafter. The type of difference found in the earlier stages

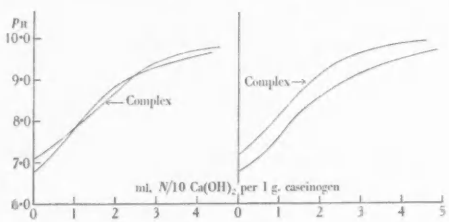

Fig. 2.

was somewhat unexpected and was perhaps partly due to the presence of small amounts of soluble phosphate which had escaped diffusion, for it was less evident on longer dialysis (*cf.* curves for 18 and 66 hours' dialysis, Fig. 2). Conclusions

drawn from the results of prolonged dialysis must be accepted with some reserve however since it is difficult completely to eliminate bacterial changes which may alter the shape of the titration curves.

It is clear from the present results however that the titration curves of the caseinogenate and its calcium phosphate complex are not identical, and that is the main point, for it indicates that the quantity of calcium associated with the protein in the complex may differ somewhat from that attached to the isolated protein at the same p_H. This finding favours then the more natural view that the complex consists of calcium caseinogenate and tricalcium phosphate, uniting probably with some slight change in reaction, rather than that which, on a strict interpretation of the synthetic experiments, would make it appear to consist of calcium caseinogenate united with a perhaps variable mixture of phosphate (chiefly tertiary) and possessing a p_H identical with that of the parent caseinogenate.

It is hoped to return to a more quantitative consideration of these and similar titration curves on a future occasion. It may be remarked here however that the differences between them provide additional evidence of the existence of a chemical union between calcium caseinogenate and calcium phosphate.

Summary.

1. Further experiments on the effect of colloidal calcium phosphate (and carbonate) on the formaldehyde titration of calcium caseinogenate are described, and are interpreted as showing the existence of some type of chemical union between the calcium caseinogenate and the colloidal calcium phosphate of milk.

2. The effect of oxalate on the titratable acidity of milk and caseinogen-calcium phosphate complexes is examined. It appears to indicate that the inorganic colloid constituent is mainly if not entirely tertiary phosphate, a conclusion supported also by the results of experiments on the preparation and electrometric titration of the complexes.

3. The results, taken together, suggest that the caseinogen-calcium phosphate of milk has the nature of a double salt of calcium caseinogenate and tricalcium phosphate.

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CXXXV. POTENTIOMETRIC STUDY OF PHOTO-FLAVIN.

By KURT GUENTER STERN.

From the Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London.

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It has previously been shown that several members of the recently described flavin or lyochrome series of pigments exhibit the properties characteristic of reversible oxidation-reduction systems [Stern, 1934, 1].

Photolysis of the flavin molecule in alkaline solution yields a pigment of smaller molecular weight which retains the colour, fluorescence and reversibility of oxidation-reduction of the parent compound and in addition is soluble both in chloroform and in water, whereas the native flavins are insoluble in chloroform [Warburg and Christian, 1932]. Following the discovery by Warburg and Christian of this peculiar reaction in the case of yeast-flavin which these authors designate "gelbes Oxydationsferment," the same process was found to occur in all other flavins which were tested, *i.e.* ovoflavin and lactoflavin (vitamin B_2) [Kuhn and Wagner-Jauregg, 1933; Kuhn, György and Wagner-Jauregg, 1933; Ellinger and Koschara, 1933], hepatoflavin, uroflavin and maltoflavin [Stern, 1933; 1934, 1].

From horse-liver fractions which had been obtained by adsorption and elution as described in the experimental part of this paper photo-hepatoflavin was obtained by the procedure devised by Warburg and Christian for yeast; 9 kg. of liver yielded thus between 6 and 10 mg. of recrystallised product. Not only were the colour, the shape and arrangement of the micro-crystals, and the other general properties of this compound obviously the same as those given by Warburg and Christian for their photo-product from yeast-flavin, but the melting-point and the spectroscopic data of these two substances were within certain limits the same. A similar agreement exists between photo-hepatoflavin and the "lumi"-lactoflavin of Kuhn, Rudy and Wagner-Jauregg [1933]. It is remarkable that the absorption spectra of the non-irradiated ovoflavin and lactoflavin are much the same as those of the different photo-flavins [Kuhn, György and Wagner-Jauregg, 1933]. It follows therefrom that the sugar-like grouping $C_4H_8O_4$ which is detached from the coloured part of the flavin molecule by the photo-reaction does not affect the light-absorption of the complex molecule. Similar observations have been reported in the study of nucleosides [Holiday, 1930].

A more detailed account of spectrographic work on hepatoflavin and photo-hepatoflavin done jointly with Dr E. R. Holiday will be presented in a separate publication. It will suffice therefore to tabulate here the molecular extinction coefficients of photo-hepatoflavin in 0·01 *N* NaOH as compared with those of photo-yeast-flavin, lumi-lactoflavin and ovoflavin, as given by Warburg and Kuhn respectively.

Table I.

Photo-hepatoflavin sublimed, in 0·01 *N* NaOH		Photo-yeast-flavin (chloroform)		Lumi-lactoflavin (chloroform)		Ovoflavin (water)	
λ	x	λ	x	λ	x	λ	x
445 $m\mu$	$1{\cdot}19.10^4$	452 $m\mu$	$2{\cdot}58.10^4$	445 $m\mu$	$2{\cdot}6.10^4$	446 $m\mu$	$2{\cdot}11.10^4$
352	$1{\cdot}49.10^4$	350	$1{\cdot}94.10^4$	360	$1{\cdot}7.10^4$	366	$1{\cdot}75.10^4$
267	$5{\cdot}89.10^4$	270	$6{\cdot}16.10^4$	270	$7{\cdot}5.10^4$	267	$8{\cdot}5\ .10^4$

$$\left(x = \text{mol. extinction coeff.} = \frac{2{\cdot}30}{c.d} \log \frac{I_0}{I}\right).$$

With sufficient amounts of the crystallised photo-hepatoflavin in hand it seemed to be of interest to undertake a study of its behaviour as an oxidation-reduction system by means of potentiometric analysis. This was done to collect data which might be useful for a comparative study of the potentials of various flavins, but especially to gain some insight into the structure of the special grouping of the molecule which is responsible for its function as a redox-system *in vitro* and probably as an intermediary redox catalyst (carrier) *in vivo*. The fact that the photo-flavins are artefacts and represent only a part of the original flavin molecule is of course not to be overlooked. But since the photo-flavins retain so many features of the native compounds and especially since they still incorporate the arrangement of atoms responsible for both colour and reversible oxidation-reduction, it may be hoped that the information obtained by this study will have a certain bearing on the problem of the flavins in general. Incidentally it was found that the system under investigation is one capable of semiquinone formation and is therefore accessible to a theoretical treatment of the experimental data along the lines recently developed by L. Michaelis and his colleagues. Kuhn and Wagner-Jauregg [1934] observed in qualitative experiments that, whereas the colour change on reduction of the flavins in neutral or alkaline solution is from yellow to colourless, in strongly acid solution it is from yellow over an intermediary red stage to colourless. They advanced the hypothesis that the red colour belongs to an organic radical of the character of a semiquinone. Certain analogies with pyocyanine, to which Kuhn referred in a recent lecture [1934], were however invalidated by the use of an erroneous value of the pyocyanine potential [Stern, 1934, 2]. Moreover there was no evidence as to the nature of the red compound.

It will be shown that the oxidation or reduction of photo-flavin in acid solution is performed in two steps each involving the exchange of one electron, a complete separation of the two processes only being effected in very acid solutions. The intermediary stage may be depicted as a semiquinone, *i.e.* a partly reduced and partly oxidised molecule of remarkable stability in view of its character.

The author was fortunate enough to secure a small sample of crystalline photo-yeast-flavin prepared by Prof. Otto Warburg and Dr Walter Christian. This sample was presented to Prof. E. C. Dodds in July 1933 and stored since then at 0°. My best thanks are due to Prof. Warburg and to Prof. Dodds for their kindness in placing this valuable substance at my disposal.

Comparative measurements at three different p_H values showed that in addition to the above-mentioned properties the potentials of the photo-flavins are also in close agreement. The conclusion seems therefore to be warranted that the photo-flavin from hepatoflavin is identical with that from yeast-flavin and most probably with the photo-products of the other flavins.

When this work was already well advanced a publication by Bierich and Lang [1934] dealing with the potentials of a yellow pigment from mammalian tissues came to our knowledge. In this paper the data given by Bierich *et al.* [1933] in a preliminary communication were somewhat extended. The authors do not describe in detail the preparation or the properties of their pigment. They state that only very small quantities of the impure pigment in solution were at their disposal. There is no mention of the spectroscopic properties. No single titration curve is reproduced and the authors refer to their curves as being "typically S-shaped" and indicating an electron number $n=2$. To corroborate this statement the index potential is given as 14·6 and 14·7 mv. The curve relating the normal potentials at varying hydrogen ion concentrations from p_H 1 to 11·4 exhibits values which differ from our values by as much as 30 mv. With the exception of one experiment all titrations performed by Bierich and co-workers were oxidative ones with ferricyanide as titrant. We find that titrations with ferricyanide at low p_H yield no curves capable of interpretation owing to the formation of a blue-green complex in acid solutions, which seems to be of irreversible nature. The authors failed to observe the red intermediate and the steepening of the titration curves in the acid range where the semiquinone is formed. At my request Prof. R. Bierich provided me with a more detailed description of the preparation of his pigment. Following this a small amount of pigment in solution was obtained from 500 g. liver. The spectrographic examination, kindly carried out by Dr E. R. Holiday, revealed an absorption spectrum very similar to that of the photo-flavins. The pigment could be reversibly reduced and oxidised and the red intermediate could be observed in mineral acid solution. It appears therefore that Bierich has been working with impure photo-flavin and that the discrepancies between his results and ours are to be ascribed to the impurity of his preparation and, as far as the acid range is concerned, to the different experimental procedures.

Experimental.

Preparation of photo-hepatoflavin.

4·5 kg. of fresh horse-liver are freed from fat and connective tissue and are finely minced in an electrical mincer. The liver mash is stirred into 6 l. of boiling tap-water and boiling is maintained for 10 minutes. After cooling to about 40° the decoction is pressed by hand through press-cloth. To the slightly turbid yellow-brown extract about 50 ml. conc. HCl and 250 g. fuller's earth are added and adsorption carried out by mechanical shaking for one hour. The remaining solution is separated from the adsorbate by means of a centrifugal filter-drum lined with press-cloth. After washing with at least 4 l. distilled water the hepatoflavin is eluted from the fuller's earth by shaking it twice with 1·5 l. elution mixture (1 l. distilled water *plus* 250 ml. methyl alcohol *plus* 250 ml. pyridine) for 1 hour. The eluate is obtained preferably by suction through a Büchner funnel in which a thin layer of kieselguhr is spread over two ordinary paper filters. The clear solutions show a deep yellow colour and a strong green fluorescence even in ordinary daylight. They are jointly concentrated *in vacuo* at approximately 40° to about 500 ml. The remaining pyridine is removed by treatment with about 1 l. ether in three portions. The watery layer is brought to an NaOH content of 0·5 *N* by the addition of 10*N* NaOH. A colourless precipitate is formed which is removed by filtration. The dark yellow solution is irradiated for at least 5 hours from a 100 c.p. metal filament lamp at 15 cm. distance. The temperature is kept below 20° by spreading the solution in a glass

dish which is immersed in a larger glass basin through which tap-water flows rapidly. The light-source is installed beneath the cooling bath. During irradiation the solution is mixed by stirring with nitrogen or air. After photolysis is completed, which may be ascertained by carrying out a chloroform test on a small sample, the solution is acidified with HCl to Congo red, whilst carefully cooled with ice-water. It is then extracted three times with three lots of about 300 ml. benzene, which removes practically no pigment. The photo-flavin is extracted by frequent extractions with small amounts of chloroform until the chloroform layers are only faintly coloured. Though the watery layer may still show some yellow colour, owing probably to non-flavin pigments, it is discarded and the combined chloroform extracts (about 1500 ml.) are filtered through a filter moistened with chloroform or are centrifuged to remove residual water and also colourless flocks. The chloroform solution is then concentrated *in vacuo* to about 300 ml. and the photo-flavin is extracted with 100 ml. 0·1 *N* NaOH. The yellow alkaline solution is acidified with HCl to Congo red and the photo-product is extracted by shaking with small amounts of chloroform. The exchange between water and chloroform phases is repeated at least three or four times. The last chloroform extract is filtered and the chloroform completely removed *in vacuo* or in a stream of cold air. The thin yellow-brown film is dissolved in dilute alkali (about 20 ml.), filtered and just acidified with HCl. Usually a faint turbidity appears. On slow concentration of the solution *in vacuo* over sulphuric acid, photo-flavin crystals gradually appear. When the volume of the solution is about one-fourth of the original the crystals are filtered off or separated from the mother-liquor by centrifuging. After washing with cold water in which they are only sparingly and slowly soluble the crystals are dried *in vacuo* over sulphuric acid. The yellow-brown needles melt with decomposition about 340° (uncorrected). They may be sublimed in a high vacuum at 230° without decomposition.

Arrangement and methods of potentiometric measurements.

In general the technique developed by Clark and by Michaelis for measuring oxidation-reduction potentials was employed. A Tinsley research potentiometer was used, the scale of which reads to 1 mv. and allows a fair estimation of 0·1 mv. Since the internal resistance of the instrument is comparatively low (40 ohms) a resistance of 1500 ohms was connected in series with the reference electrode and titration electrodes. As indicator a Tinsley deflection galvanometer of the lamp and scale type was used. The sensitivity was given by the maker as 100 scale divisions per milliamp. The period was one second. As normal element a cadmium cell was used that had been calibrated by the Physikalisch-Technische Reichsanstalt, Berlin (1019·3 mv.). The actual titration outfit consisting of a saturated calomel electrode, and the titration vessel was placed in a spacious air-thermostat that was kept at 30·0 ± 0·5°. The volume of the titration vessel was 30 ml., the volume of liquid necessary to cover the electrodes completely was 10 ml. As experimental electrodes one gold-plated and one bare platinum electrode were used. The titrant was delivered from a micro-burette holding 1 ml. and graduated in 0·01 ml. The calomel electrode was frequently standardised by means of the hydrogen electrode in standard acetate and gave almost invariably a potential difference against the latter of 515 to 515·5 mv. For the measurement of $[H^+]$ three methods were employed alternately and sometimes two of them were applied to the same solution: (*a*) measurement of p_H with the U-hydrogen electrode of Michaelis; (*b*) reduction of the buffered pigment solution by colloidal palladium and hydrogen and estimation of $[H^+]$ from the potential values of the experimental electrodes,

previous to oxidative titration with ferricyanide [Michaelis and Eagle, 1930]. It was found unnecessary to provide for a separate platinum electrode covered with platinum black, since after a sufficient time of hydrogenation both the gold-plated and the bare platinum electrodes gave identical and true potential values as controlled with method (*c*). This may be due either to incomplete smoothness of the electrode surfaces or to the presence of the colloidal palladium in the system which might form a small deposit on the electrodes and make them act as palladium electrodes which are known to be perfect hydrogen electrodes[1]. (*c*) The standard method, however, employed in the present study was the measurement with the glass electrode arrangement as developed by Greville and Maclagan [1931] at this Institute. I am indebted to Mr M. E. H. Fitzgerald for these measurements.

The hydrogen and nitrogen used for the titration experiments were taken from cylinders containing commercial gases of 99·9 % purity. Complete purification was achieved according to Michaelis [1931, 1]. A 5 m. copper tube was used to lead the purified gases into the titration vessel. This ensured a rapid cooling of the gas heated to approximately 500° in the furnace. Even in the most negative potential ranges no drift of the potentials towards the positive range which would indicate the presence of traces of oxygen could be observed.

The electrometric titrations for ascertaining the position of the dissociation constants were performed in the same apparatus with the exception of the electrodes used. Two platinum wires hammered flat at the end and covered with a platinum black deposit of medium thickness were submerged in 12 ml. solution containing 2 mg. (10^{-5} mol.) recrystallised photo-hepatoflavin *plus* 0·1 ml. palladium sol. The titrant was 0·01 *N* NaOH. Throughout the experiment the pyrex titration vessel was flushed with a stream of pure hydrogen at 30°. Whereas the dissociation constant of the reduced form of the pigment could be determined in this way, an attempt to apply this technique (with the omission of the palladium sol) to the oxidised form failed owing to a complete disagreement of the electrodes and weakly maintained potentials. This may have been caused by the competition for the electrode surface between the oxidation-reduction system and the hydrogen ions present in the solution.

Results.

Titration experiments on photo-hepatoflavin.

To permit an adequate description of this system 26 titration experiments were performed at varying hydrogen ion concentrations, the limits being p_H −0·2 and 12·4. The molar concentration of the photo-flavin solutions was of the order 10^{-3} to 10^{-4}. Except in the very acid range where HCl and H_2SO_4 were used, buffers of sufficient strength (0·5 to 0·1 *M*) were employed to ensure a constant $[H^+]$ during the experiment. The p_H measurements with the glass electrode were usually made after the titration was finished.

The first series of titrations dealt with the behaviour of the system in the neutral and alkaline ranges. Here the colour change is from pure yellow to colourless on reduction and *vice versa* on oxidation of the leuco-compound. In this range the system behaves exactly like one of the well-known organic redox-indicators, *e.g.* methylene blue. The electrodes adjust themselves almost instantaneously to the potential changes brought about by the variation of the oxidant/reductant ratio on addition of titrant, so far as the range from 10 to 90 %

[1] The possibility of determining p_H in this way was discovered by Biilman [(1927) *Z. physikal. Chem.* **130**, 566]. I am indebted to Prof. L. Michaelis for pointing this out.

reduction is concerned. Here the agreement between the gilded and bare platinum electrodes is perfect within 0·5 and many times within 0·1 mv. No perceptible potential drift is observed up to 30 minutes. Current of the strength limited by the resistance of the chain may be drawn from the electrodes for several seconds without upsetting the potential readings or causing polarisation. Outside the range of sufficient poising the potentials are established only after some time—10–40 minutes—has elapsed since the addition of the titrant. In this range the agreement between the different electrodes is usually less satisfactory and varies between 1 and 10 mv. It may be remarked that the same applies to the best reversible systems known. The slope of the titration curves in the range from p_H 6 to 12·4 is that of an ordinary two electron system with an index potential in the neighbourhood of 14 mv., within the limits of experimental inaccuracies and the error involved in the graphical interpolation for the determination of the mid-point of the curves. The latter is almost unavoidable in view of the fact that even in the case of the photo-flavin where the end of the titration is indicated by a pronounced jump of potential the selection of the actual end-point invariably introduces a certain arbitrary element[1].

In the indicated p_H range the experimental method exclusively used was that of Michaelis and Eagle [1930], consisting in the reduction of the system by means of hydrogen activated by Paal's colloidal palladium and by reoxidising the leuco-compound with ferricyanide. The ferricyanide used was of analytical purity and the strength of the solution was determined in two instances by iodimetric titration. The solvent was de-aerated before addition of the accurately weighed oxidant by a stream of pure nitrogen, and the solution as well as the amount transferred to the micro-burette were protected against oxygen by a thick layer of liquid paraffin.

The curves thus obtained between p_H 7·4 and 12·4 have been collected in Fig. 1 after transformation of the amounts of oxidant used into the corresponding degree of oxidation.

The best proof of the strict reversibility of the system under investigation is perhaps to be seen in the fact that several of the titration curves shown in this and also in the following diagram were obtained with the same specimen of photo-flavin solution, the p_H of which was adjusted to another value under electrometric control after each experiment was finished.

The second series of titrations deals with the phenomena in acid solutions. When attempts were made to apply the technique outlined above, which had given such satisfactory results in the neutral and alkaline ranges, to solutions of photo-flavin of $p_H < 6$ unexpected difficulties were met. At p_H 5·6 it was still possible to obtain an S-shaped curve as shown in Fig. 2, but the slope of the curve was much steeper in the first half of the experiment than in the second half. In the range below p_H 5 we did not succeed in obtaining S-shaped curves by means of the oxidative technique. With ferricyanide as oxidant non-characteristic curves were recorded. The potentials showed a rapid drift towards the positive side especially as the titration proceeded. No definite end-point corresponding to a definite jump of potential was reached in repeated attempts. With decreasing p_H values the appearance of a blue-green colour was noticed

[1] It should be remarked, however, that the conclusion concerning the nature of the process as a two-electron system has been derived from the slope of the titration curves, *i.e.* from the value of the index potential. The experimentally found position of the end-point fell invariably short of the stoichiometric equivalence point by as much as 50 and even 60 %. This holds true even on titration of photo-flavin purified by sublimation. This discrepancy is the object of further investigation.

as the titration proceeded. It became most pronounced when p_H 0 was approached. The colour persisted after admission of oxygen. On treating with chloroform no pigment could be extracted, which proves that the photo-flavin was actually transformed into a different compound. Sodium amalgam brought about a decoloration in mineral acid solution, which was apparently irreversible since no colour returned on vigorous shaking with air. It is planned to investigate this matter more thoroughly. At present the evidence is sufficient to warrant the statement that ferricyanide is obviously useless as a titrant in the acid range.

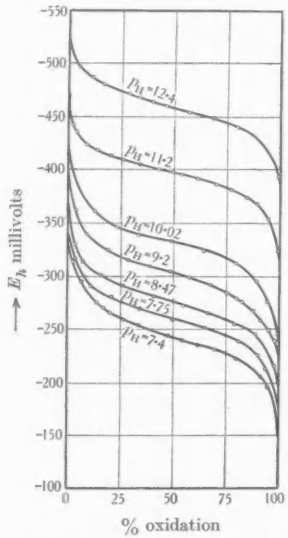


Fig. 1. Titration curves of photo-hepatoflavin at p_H 7·4 to p_H 12·4.

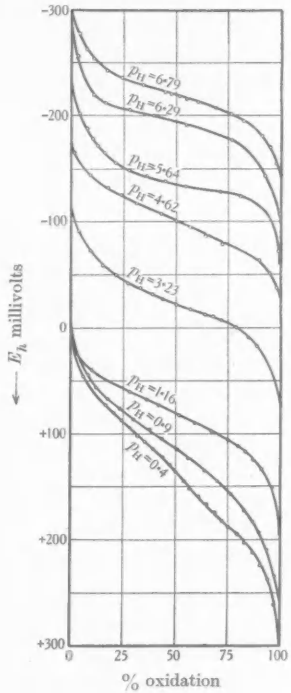


Fig. 2. Titration curves of photo-hepatoflavin at p_H 0·4 to p_H 6·79.

This is important in view of Bierich and Lang's [1934] assertion that ferricyanide may be used throughout the whole p_H range. Since attempts with dichromate and benzoquinone as oxidants at p_H 3·98 and 4·62 respectively did not yield quite satisfactory results either, it was suspected that the reduction procedure with palladium and hydrogen preceding the titrations might lead to different reduction products in acid and alkaline solutions respectively, more especially since it is known that hydrogenation of flavins may lead to the absorption of 1 and also of 3 mols of hydrogen per mol. flavin according to the experimental conditions chosen [Warburg and Christian, 1933; Kuhn, György and Wagner-Jauregg, 1933]. The following experiment was therefore devised and performed in co-operation with Mr G. D. Greville of this Institute. 0·7 mg. of a repeatedly

recrystallised sample of photo-hepatoflavin was dissolved in 3·5 ml. distilled water containing 0·2 ml. *N* NaOH. 1 ml. of this solution (corresponding to 0·2 mg. of photoflavin) was transferred to the main compartment of each of three Warburg vessels of the conical type. The volume of the solutions was made up to 2·0 ml. by addition of water and HCl (0·2 *N*) to adjust the p_H to 11·6, 5·17 and 1·74 respectively, as determined with the glass electrode after the experiment. KOH was used for absorption of CO_2 which might possibly be evolved. The side-bulbs contained 0·1 ml. colloidal palladium solution. An atmosphere of pure hydrogen was established by flushing the vessels attached to the manometers for 10 minutes with hydrogen which had passed the quartz copper furnace. After equilibration in the Warburg thermostat at 37° the palladium sol was tipped in from the side-bulb and the H_2 absorption followed for 125 minutes. A similar experiment was conducted with the omission of the photo-flavin. After subtraction of the hydrogen absorbed in this control experiment it was found that the photo-flavin had absorbed 14 μl. of hydrogen at p_H 11·6, 15 μl. at p_H 5·17, and 18·5 μl. at p_H 1·74. These hydrogen amounts

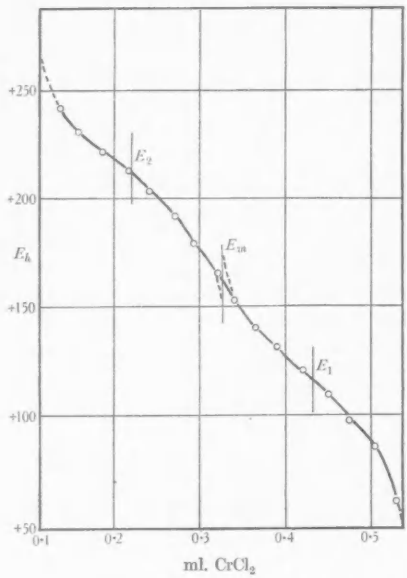


Fig. 3. Titration of photo-hepatoflavin with $CrCl_2$ at p_H – 0·2.

may probably be regarded as identical within the limits of error. Calculated per mg. photo-flavin an absorption of 70 to 92·5 μl. hydrogen was therefore observed as compared with 87 μl. absorption found by Warburg and Christian in weakly alkaline solution under similar conditions. The inference is that the difficulties encountered in the so-called oxidative titrations have their origin in the oxidative and not the reductive phase of the experiment.

Further work in the acid range was therefore conducted by exclusive adoption of the reductive titration method. Hydrosulphite, titanous chloride and

chromous chloride were used. Between p_H 5 and 0·4 titanous chloride proved to be very satisfactory. At still lower p_H the reductive power of this reagent was insufficient to bring about a definite jump in potential at the end of the titration owing to the approach to the hydrogen potential. Here chromous chloride with its extreme reductive power corresponding to a potential of hydrogen overvoltage was successfully used.

As will be seen from the composite diagram in Fig. 2 the slope of the titration curves becomes increasingly steeper with increasing $[H^+]$ below p_H 5. From p_H 4 to 1 the character of the curves is approximately that of a one electron system, the index potential varying between 20 and 25 mv. At $p_H < 1$ the steepness gradually increases until at p_H 0·4 the curve loses its uniform shape and two maxima appear. At this point evidently a separation in two steps of reduction is becoming manifest. On the titration of photo-flavin solutions below p_H 5 an intermediate orange tint is noticeable on reduction. With increasing $[H^+]$ this intermediate colour becomes gradually more and more pronounced until, as observed for the first time by Kuhn and Wagner-Jauregg [1934], a pure red appears midway between the yellow colour of the fully oxidised form

Table II.

Titration of crystalline photo-hepatoflavin in N HCl with $5 \cdot 10^{-3}$ M titanous chloride: Temp. 30°. p_H 0·4. E (potential difference between calomel and normal H_2 electrode) 0·2365 v.

(Calomel/standard acetate H_2 electrode 0·515 v.)

$TiCl_3$ ml.	Gold-plated Pt-electrode	Bright Pt-electrode	Mean	E_h	$E_{\frac{1}{4}}$ $E_{\frac{1}{2}}$ $E_{\frac{3}{4}}$	Colour	Remarks
0·00	+0·234	+0·2315	+0·2327	+0·4692		Lemon-yellow	Nitrogen
0·03	0·1755	0·1745	0·175	0·4115			
0·06	0·072	0·077	0·0745	0·311		Pure yellow	
0·095	0·022	0·0288	0·0254	0·2619		Yellow with	
0·125	−0·001	0·003	0·001	0·2375		pink shade	
0·165	0·0135	−0·0102	−0·0118	0·2247		Pink-yellow	
0·195	0·0245	0·0225	0·0235	0·213			
0·23	0·033	0·032	0·0325	0·204		Red-yellow	
0·265	0·0405	0·040	0·04025	0·1933	$E_{\frac{3}{4}}$	Yellow-red	
0·30	0·0475	0·465	0·047	0·1895	0·187		
0·335	0·0545	0·054	0·0542	0·1823			
0·365	0·0618	0·061	0·0614	0·1751		Red	
0·40	0·069	0·0688	0·0689	0·1676		↓	
0·435	0·0768	0·076	0·0764	0·1601			
0·47	0·0855	0·085	0·0852	0·1513	$E_{\frac{1}{2}}$	↓	
0·50	0·0925	0·092	0·0922	0·1443			
0·54	0·105	0·0995	0·100	0·1365	0·135	Red colour	
0·575	0·108	0·107	0·1075	0·129		gradually	
0·605	0·115	0·114	0·1145	0·122		fading	
0·645	0·122	0·121	0·1215	0·115		↓	
0·685	0·1285	0·1275	0·128	0·1085			
0·715	0·1359	0·1345	0·1352	0·1013	$E_{\frac{1}{4}}$		
0·75	0·1435	0·1425	0·143	0·0935	0·90		
0·785	0·1505	0·149	0·1497	0·0868		Pale pink	
0·82	0·1585	0·1565	0·1575	0·079			
0·855	0·167	0·1643	0·1657	0·0708			
0·96	0·1925	0·189	0·1905	0·046		Colourless	
	0·215	0·210				10 mins.	Palladium-hydrogen
	0·261	0·261				13	
	0·262	0·2625				35	
	0·2628	0·2628	0·2628			45	
	p_H calculated from this value						

Table III. *Summary of titration experiments performed on photo-hepatoflavin*[1].

Exp. No.	p_H	Buffer	Method	$E_{\frac{1}{4}}$	$E_0'=E_{\frac{1}{2}}$	$E_{\frac{3}{4}}$	E_i	Appar. n	0·03 log K $=E_M-E_1$	log K
29	−0·2	3 *N* H_2SO_4	Red. : $CrCl_2$	+0·106	+0·152	+0·202	0·048	0·6	+0·047	+1·6
26	+0·4	*N* HCl	Red. : $TiCl_3$	+0·090	+0·135	+0·187	0·048	0·6	+0·047	+1·6
25	0·9	Citrate-HCl	,,	+0·080	+0·113	+0·152	0·036	0·8	+0·03	+1
24	1·16	,,	,,	+0·058	+0·082	+0·105	0·0235	1·25	+0·006	+0·2
17	3·23	,,	Red. : $Na_2S_2O_4$	−0·044	−0·023	−0·003	0·0205	1·4	−0·006	−0·2
21	4·62	Acetate	,,	−0·124	−0·100	−0·074	0·025	1·15	+0·012	+0·4
2	5·64	Phosphate	Oxid. K_3FeCy_6	−0·149	−0·135	(−0·127)	0·014	2	−0·06	−2
6	6·29	,,	,,	−0·206	−0·194	−0·179	0·013	2·1	−0·06	−2
7	6·79	,,	,,	−0·234	−0·218	−0·202	0·016	1·75	−0·03	−1
1	7·41	,,	,,	−0·260	−0·243	−0·228	0·016	1·75	−0·03	−1
8	7·75	,,	,,	−0·275	−0·258	−0·242	0·0165	1·7	−0·03	−1
5	8·47	,,	,,	−0·293	−0·275	−0·257	0·018	1·55	−0·018	−0·6
12	9·2	Phosphate-NaOH	,,	−0·318	−0·303	−0·285	0·0165	1·7	−0·03	−1
3	10·02	,,	,,	−0·344	−0·332	−0·315	0·0145	1·95	−0·06	−2
23	11·2	,,	,,	−0·411	−0·397	−0·382	0·0145	1·95	−0·06	−2
10	12·4	,,	,,	−0·473	−0·458	−0·445	0·014	2	−0·06	−2

[1] Potential values referring to the normal H_2 electrode.

and the colourless leuco-compound. This occurs at $p_H > 1$–2. An almost complete separation of the two steps is reached at p_H −0·2 as shown in Fig. 3. Table II contains the protocol of the experiment at p_H 0·4, the corresponding curve of which is the lowest curve in Fig. 2. Maximum formation of the intermediate red colour thus occurs between the first quarter and the mid-point of the titration curve. Table III contains the data of the 16 experiments which correspond to the diagrams.

The foregoing observations and the fact that all the changes reported in colour and potential on titration of acid solutions of photo-flavin are fully reversible leave no doubt that the red intermediate is not a secondary reaction product but belongs to the group of quinhydrone-like substances which includes both the meriquinones and the semiquinones. According to Willstätter and Piccard [1908] meriquinones are molecular compounds composed of the oxidised and reduced forms of the same substance in which the ratio between the components need not necessarily be unity. The semiquinones on the other hand are molecules of partly reduced and partly oxidised character with an odd electron number and therefore of the nature of an organic radical, as depicted by Weitz [1928]. Though the existence of true meriquinones cannot be denied on the ground of mere theoretical considerations, potentiometric analysis by Michaelis and his colleagues and independently by Elema [1931; 1933] has in all cases tested led to the characterisation of such intermediates as semiquinones.

A survey of the experimental data of the present study leaves little doubt that the red intermediate in the photo-flavin reduction also belongs to the group of semiquinones. This is already evident from the fact that each of the two separate steps of the titration curve as obtained at p_H 0·4 or −0·2 is symmetrical in relation to the respective mid-point. The second criterion given by Michaelis, namely the independence of the value of the normal potentials of the two steps of the volume of the system, could not be tested owing to the scanty solubility of the photo-flavin in ranges of very low p_H and also to the small amounts of the compound at present available.

For the theory and mathematical analysis of the two-step oxidation with an intermediary semiquinone formation the reader is referred to the original

papers of Michaelis [1931, 2, 4; 1932] and also to the monograph by that author [1933]. For our purpose it may suffice to state that the values of the potentials recorded on titration of such a system are defined by the three equations:

$$E=E_m+\frac{RT}{2F}ln\frac{t}{r};\quad E=E_1+\frac{RT}{F}ln\frac{s}{r};\quad E=E_2+\frac{RT}{F}ln\frac{t}{s};$$

where E_m is the potential in the mid-point of the whole titration curve, E_1 the normal potential of the first and E_2 of the second step; t designates the concentration of fully oxidised (holoquinoid), r that of the fully reduced (benzenoid), and s that of the semiquinone stage. The distance of E_1 and of E_2 from E_m equals the actual distances $E_{\frac{1}{4}}-E_{\frac{1}{2}}$ and $E_{\frac{3}{4}}-E_{\frac{1}{2}}$ respectively as taken from the curve only if a complete separation of the single steps has been achieved. In the more frequent case of an overlapping of the single curves the distance of E_1 and E_2 from E_m may be estimated from the index potential by the aid of a table constructed by Michaelis [1933]. These values are given in the last but one column of Table III. The dismutation of the semiquinone into the holoquinoid and benzenoid forms proceeds according to the equation $s^2=krt$, where the mass action law constant k is called the formation constant of the semiquinone. Taking into consideration the dependence of degree of dissociation of the components of the system upon p_H, k also becomes a function of p_H and is designated then as the apparent or effective formation constant of the semiquinone. Its value is a measure of the probability and stability of semiquinone formation under a given set of experimental conditions. It is an empirical rule that semiquinone formation becomes manifest with increasing $[H^+]$. The last column of Table III contains the value of log k at varying p_H values.

Fig. 4 represents the essence of the present study. It gives in the fully drawn line the graphically determined mid-points of all titration curves plotted against p_H. This curve shows three inflections near p_H 0·4, 7·75 and 10. Between

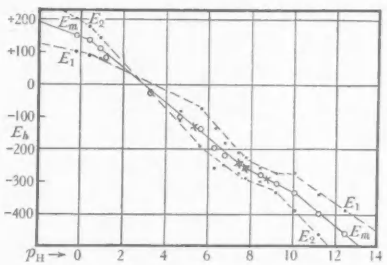


Fig. 4. Normal potentials of the holoquinoid, semiquinoid and benzenoid form of photo-flavin at varying $[H^+]$, referred to the normal hydrogen electrode at 30°. ⊙ Measurements on photo-hepatoflavin; × measurements on photo-yeast-flavin.

p_H 0·4 and 7·7 and also between 10 and 12·4 we find a rough approximation to a "60 mv. slope," whereas between −0·2 and +0·4, and also between 7·7 and 10 the slope is a "30 mv." one. This would mean that near p_H 0·4 and 10 are situated dissociation constants of the oxidised form and near p_H 7·7 one of the reduced form. The dotted lines represent the corresponding curves of E_1 and E_2 which intersect somewhere between p_H 1 and 4. Visible semiquinone formation of appreciable extent is obviously limited to the range of $p_H<2$.

Only here may the position of the normal potentials of the two steps be charted with confidence, whereas in the lower part of the E/p_H curve an uncertainty in the index potential of 1 mv. already greatly influences their position.

The following two features of the photo-flavin as a reversible redox system appear to be worthy of special mention: (1) the position of the normal potential at p_H 7 in a comparatively very negative region, namely at -227 mv., ranging on the scale of known systems between 1:5-anthraquinonedisulphonate (E_0' at p_H 7: -200 mv.) and β-anthraquinonesulphonate ($E_0' = -250$ mv.) [*cf.* Stern, 1934, 3]; (2) the remarkable stability of all the three possible forms of photo-flavin, *i.e.* of the holoquinoid, semiquinoid and benzenoid modifications.

The position of a dissociation constant of the fully reduced form near p_H 7·7 could be independently demonstrated by electrometric titration with NaOH in the manner described on p. 953.

Titration experiments on photo-yeast-flavin.

Three oxidative titrations on solutions of the crystalline photo-product of the yellow oxidation enzyme from yeast (Warburg) with palladium-hydrogen as reductant and ferricyanide as oxidant were performed at p_H 5·34, 7·4 and 8·73 respectively. The result of the experiment at p_H 7·4 is shown graphically in Fig. 5. The E_0' as taken from this graph is -244 mv. and is therefore identical

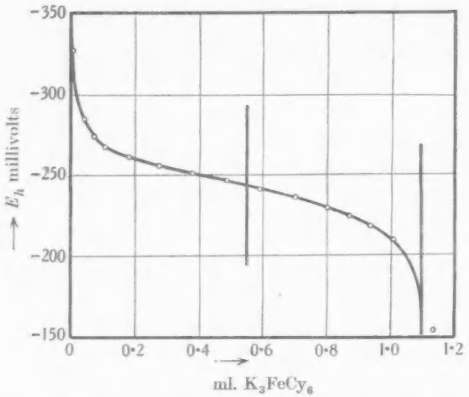


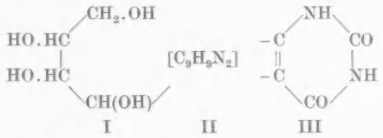
Fig. 5. Curve obtained on oxidative titration of the photo-product of the yellow oxidation enzyme (Warburg) at p_H 7·4. $E_0'' = -0{\cdot}244$ mv.

with the corresponding value for photo-hepatoflavin at the same p_H (-243 mv.) within the limits of experimental error. The values obtained for the normal potentials at p_H 5·34 and at 8·73 are represented as crosses in the main diagram Fig. 4. It may be seen that these values are situated on the E_0'/p_H curve for the photo-hepatoflavin.

This fact is further and independent evidence that the two photo-flavins (and very probably all the known photo-flavins) are identical.

Discussion.

To the native flavin molecule (but without the colloidal bearer to which the flavin may be attached as a prosthetic group) the following tentative formula has been assigned by Kuhn, Rudy and Wagner-Jauregg [1933]:



I II III

In the course of the photo-reaction, formulated by these authors as

$$C_{17}H_{20}O_6N_4 - C_4H_8O_4 = C_{13}H_{12}O_2N_4,$$

the sugar-like portion I in the above formula is split from the coloured structure II+III, which then represents the photo-flavin molecule. About the portion II little is known except that it is relatively stable and contains two weakly basic N-atoms which are probably of tertiary nature and share the chromophoric double bonds.

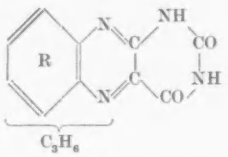
It may be tempting to associate the presence of the alloxan ring (III) with the function of the flavin molecule as a reversible redox system. This idea must however be abandoned. The alloxan-dialuric acid system as studied by Richardson and Cannan [1929] presents no analogies with the flavin system. Apart from the widely different potential values, the alloxan-dialuric acid system becomes unstable at $p_H > 5$, probably because of the isomerisation of alloxan into alloxanic acid. Furthermore the quinhydrone-like compound alloxantin is completely dissociated into alloxan+dialuric acid in the very range where semi-quinone formation takes place in the flavin system. Whereas stabilisation of the alloxantin potentials in dilute solution requires the addition of iron salts, which leads to the formation of an iron complex acting as an electro-active intermediate between the components of the main system and the electrode [Hill and Michaelis, 1933], in the case of photo-flavin complex formation with an iron compound has been shown above to set up an irreversible system.

On alkaline hydrolysis the photo-flavin molecule is split into urea and an unknown acid: $C_{13}H_{12}O_2N_4 + 2H_2O \rightarrow CO(NH_2)_2 + C_{12}H_{12}O_3N_2$ [Warburg and Christian, 1933; Kuhn, Rudy and Wagner-Jauregg, 1933]. This acid yields on thermal decomposition $CO_2 + C_{11}H_{12}ON_2$. Kuhn and co-workers compare the formation of the acid $C_{12}H_{12}O_3N_2$ from the photo-product ("lumiflavin") with that of 2-hydroxyquinoxalin-3-carbonic acid from alloxazin:

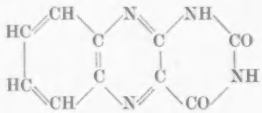
$$C_{10}H_6O_2N_4 + 2H_2O \rightarrow CO(NH_2)_2 + CO_2 + C_8H_6ON_2 \text{ [Kühling, 1891].}$$

Though Kuhn, after preliminary experiments, rejected the idea that the flavins may be constructed similarly to the alloxazin model [Kuhn and Wagner-Jauregg, 1933] he states in a later paper that on reduction in acid solution alloxazin yields a red intermediate which is similar to the flavin intermediate. It is known that even the simple quinoxalin shows the formation of a brightly coloured intermediate which is blue in this case, on reduction in acid solution [Hinsberg, 1884]. That the alloxan ring of the flavin should be contained in a purine skeleton is unlikely in view of the fact that the extinction coefficient of the short ultra-violet absorption band of the flavin is of a higher order of magnitude than that of any purine compound so far known (private communi-

cation from Dr E. R. Holiday). There seems therefore to exist some presumptive evidence in favour of a formulation of photo-flavin as follows:



This would place the flavins in the alloxazin series[1]. We find that the simplest representative of this class, namely, alloxazin itself,



exhibits even more similarities with photo-flavin than were mentioned by Kuhn. Its solution in dilute sodium carbonate is green-yellow and shows a green fluorescence. Addition of sodium hydrosulphite or titanous chloride causes slow decoloration. The red intermediate in mineral acid solution is formed not only by reduction with zinc but also by any strong reductant, *e.g.* chromous chloride. The red intermediate is obtained also on re-oxidation of the leuco-compound with air and shows a remarkable tendency to persist under aerobic conditions. A comparative study of the oxidation-reduction behaviour of alloxazin and photo-flavin and especially of the effect of substitution in the three ring systems on the potentials and on the spectrum is being undertaken in this laboratory.

The structural relationship of such a molecule as that suggested above to

Table IV.

Compound	Colour of Holoquinone	Colour of Semiquinone	E_0' at p_H 7
Hallachrome [Friedheim, 1933]	Red, p_H <8·53 Green >8·53	Brown (?)	+0·022 v.
α-Hydroxyphenazine [Michaelis *et al.* 1932]	Orange	Green	−0·07 v.*
Pyocyanine (α-hydroxy-*N*-methylphenazine) [Friedheim and Michaelis, 1931; Elema, 1931]	Red, p_H <4·9 Blue >4·9	Green	−0·034 v.
Chlororaphine (phenazine-α-carbonamide) [Elema, 1933]	Yellow	Green	−0·120 v.
Photo-flavin	Yellow	Red	−0·227 v.
Rosinduline 2 G [Michaelis, 1931, 3]	Red	Violet	−0·281 v.
Viologens (*NN'*-disubstituted *γγ'*-dipyridyliumchlorides) [Michaelis and Hill, 1933]	Colourless	Blue-violet	−0·4 v.

* From the diagrams reproduced by Michaelis *et al.* [1932, see also Michaelis 1933] it would appear that the E_0' of α-hydroxyphenazine at p_H 7·0 is −0·17 v. This value however refers to the calomel and not to the normal hydrogen electrode as stated in the text. [Private communication by Prof. L. Michaelis.]

[1] *Note added to proof.* This hypothesis has been rendered very probable by synthetic studies in the alloxazine series [Stern and Holliday (1934), *Ber. deutsch. chem. Ges.* **67**, 1104].

the phenazine pattern is quite obvious. Kuhn [1934] has already pointed out several analogies in their respective oxidation-reduction behaviours between the flavins and pyocyanine (α-hydroxy-*N*-methylphenazine). It occurs to the writer that an even more striking similarity exists between the flavins and rosinduline 2 G, especially in view of the fact that the normal potentials of this compound are close to those of photo-flavin (E_0' at p_H 7 −281 mv.) [Michaelis 1931, 3]. It should be mentioned however that the colour of the semiquinoid forms of simpler phenazine derivatives is green in contrast to the red semiquinone of photo-flavin. But the example of rosinduline where the semiquinone is violet demonstrates that the rule that the intermediates of phenazine compounds are green does not hold for the more complex derivatives.

This discussion may be concluded by a compilation of some properties of a few systems capable of semiquinone formation (Table IV).

Summary.

The behaviour of photo-hepatoflavin as an oxidation-reduction system has been studied by means of potentiometric analysis. Titrations were performed between p_H −0·2 and 12·4. Photo-hepatoflavin represents a perfectly reversible redox system, the normal potential E_0' of which is comparatively negative, namely −0·227 v. at p_H 7·0, referred to the normal hydrogen electrode.

In the neutral and alkaline ranges the titration curves are similar to those of a common organic redox indicator with an electron number of two. In the acid range the slopes of the curves become increasingly steeper with increase of $[H^+]$. At very low p_H values a break in the curves appears indicating that the reduction and oxidation are taking place here in two distinct steps each involving the exchange of one electron. The intermediate stage is characterised by a bright red colour (Kuhn). The observations point strongly to a semiquinone structure for this intermediate. A chart is given illustrating the relation between p_H and the normal potentials of the different forms (holoquinoid, semiquinoid, benzenoid form) of photo-flavin. All three forms show a fair degree of stability. The existence of appreciable amounts of the semiquinone is limited to the p_H range below 2.

The theory of Michaelis on two-step oxidation with intermediary semiquinone formation has been applied to the present system; and the distance of the normal potentials of the single steps from the mid-point found on titration, and also the effective formation constant of the semiquinone, have been calculated by means of Michaelis's equation.

Experiments carried out with the corresponding photo-product of the yeast-flavin (Warburg's yellow oxidation enzyme) yielded confirmatory evidence for the identity of the photo-flavins.

From this study several conclusions have been drawn concerning the structure of photo-flavin and especially as to the nature of the grouping of the molecule responsible for its properties as a reversible redox system. The inference is that the alloxan-dialuric acid equilibrium cannot correspond to the system set up by photo-flavin and its reductants, although the presence of an alloxan ring has been demonstrated in the molecule by Warburg and by Kuhn. A purine structure is likewise improbable. Presumptive evidence is brought forward for a structural relationship between photo-flavin and the alloxazin series as first discussed but later denied by Kuhn. This would explain certain common features of photo-flavin and phenazine derivatives, especially rosinduline, *e.g.* the negative range of potential and the phenomenon of semiquinone formation.

The author wishes to thank Prof. E. C. Dodds for his interest and encouragement. Furthermore he wishes to thank him and the Council of the Middlesex Hospital Medical School for the hospitality afforded. Sincere thanks are also due to Prof. L. Michaelis who was good enough to read the MS and to advance helpful criticisms.

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CXXXVI. STUDIES IN FAT METABOLISM IN THE FOWL.

I. THE COMPOSITION OF THE EGG FAT AND DEPOT FAT OF THE FOWL AS AFFECTED BY THE INGESTION OF LARGE AMOUNTS OF DIFFERENT FATS.

By ETHEL MARGARET CRUICKSHANK.

Poultry Nutrition Section, Animal Nutrition Institute, School of Agriculture, Cambridge.

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The study of the formation and utilisation of fat in the fowl is of fundamental interest, since our knowledge of these processes in the bird is very meagre, investigations of fat metabolism having been so far carried out almost exclusively on mammals. With a view to eliciting information along these lines it was decided to investigate two problems which presented themselves as being of primary importance to the poultry industry. (1) The approximate composition of the mixed fatty acids of the normal egg and the extent to which this may be modified by the inclusion of fat in the ration. (2) The influence of ingested fat on the composition of the depot fat. The latter investigation is of a preliminary nature.

In the following paper the two problems outlined will be dealt with in separate sections.

SECTION I.

Effect of ingested fat on the composition of the egg fat.

The data available for the egg fat concern chiefly the neutral fats or the phosphatides and not the mixed fatty acids of the entire yolk. The ether extract of the fresh yolk is about 30–32 %, a figure that appears to remain relatively constant, but variable results have been obtained for the phosphatide content, *e.g.* Mangold [1931], 4 %; McCollum *et al.* [1912], 9·4 %; Manasse [1906], 9–10 %; Serono and Palozzi [1911], 11–12 %.

The discrepancy between these results is probably due to the fact that no satisfactory method for estimating the amount of phosphatide present has yet been evolved, but it is possible also that the phosphatide content may be dependent on certain variable factors.

Many workers have investigated the composition of egg-lecithin with regard to its different components. References to this are given by MacLean [1918] and Needham [1931]. Cousin [1903], working chiefly on commercial samples of egg-lecithin, estimated the proportions of the different fatty acids present as follows: palmitic, 28·5 %; stearic, 14·2 %; oleic, 33 %; linoleic, 24 %. Levene and Rolf [1922] and Hatakeyama [1930] both recorded the presence of arachidonic

acid, and stated that linoleic acid was present only in small amount. The former found that, in contrast to liver-lecithin, egg-lecithin contained only a small proportion of highly unsaturated acids. Sueyoshi and Furukubo [1931], working with lecithin carefully purified from fat and kephalin, found that the saturated acids consisted mainly of *iso*palmitic acid, with only small amounts of palmitic and stearic acids. The proportions of the unsaturated acids present, calculated from their bromination products, were as follows: oleic, 73·2 %; clupanodonic, 5·1 %; linoleic, 2·1 %.

The results obtained for the composition of the neutral fat by the earlier workers [Liebermann, 1888; Kitt, 1897; Serono and Palozzi, 1911], present wide variations and are subject to criticism on account of the technique employed. The only detailed quantitative analysis of the egg fat available is that of Grossfeld [1933], who examined the mixed fatty acids obtained from the egg by the following method. Egg-yolks were treated with warm hydrochloric acid and extracted with light petroleum. The solution was freed from acid and water and the oil obtained by evaporation. It contained 5·1 % of unsaponifiable material and 90·7 % of mixed fatty acids, of the following composition: higher saturated fatty acids (permanganate method) 34·6 %; solid saturated fatty acids (lead salt-alcohol method) 32·4 %; stearic acid 2·2 %; palmitic acid 32·4 %; oleic acid 44·2 %; linoleic acid 18·0 %; linolenic acid (Kaufmann) 3·2 %. Butyric and *n*-octanoic acids were absent.

Various investigators have ascertained that the composition of the egg fat is affected by feeding. Henriques and Hansen [1903] found that on a carbohydrate diet the iodine value of the neutral fat averaged 78·8. With linseed feeding it rose to 97 and with hempseed to 119–123. Although the neutral fat was affected by the feeding, the fatty acids of the lecithins remained unchanged. This finding was not confirmed by McCollum *et al.* [1912] who found that the iodine value of the neutral fats varied from 52 on an approximately lipoid-free diet to 64 on an ordinary ration, while the iodine value of the lecithins on the former was 35, and on the latter 63. Similar results were obtained by Terroine and Belin [1927].

It thus appears that there is comparatively little reliable information available as to the composition of the egg fat, and though it has been ascertained that it may be affected by the nutrition of the fowl, yet we have no knowledge with regard to the changes which take place in the proportions of the component fatty acids.

Experimental.

In the experiments to be described, Light Sussex hens were kept under ordinary commercial conditions with access to free range. The control ration, which was the ordinary stock ration used on the farm, consisted of bran, maize, sharps and Sussex ground oats, supplemented by 7 % extracted soya bean meal, 7 % fish meal, and 3 % mineral mixture. The experimental groups (with one exception) received the control ration with the addition of high percentages of fats of varying composition.

The effect of ingested fat was studied (*a*) on successive eggs of individual hens, alteration in iodine value being taken as indication of a change in composition, and (*b*) on composite samples of eggs taken from groups of birds receiving different types of fat in the ration.

Under (*a*) the effects of a relatively saturated fat, *viz.* mutton fat (incorporated in the control ration at a 28 % level) and a highly unsaturated oil, *viz.* hemp oil (fed in the form of hempseed alone) were investigated. The birds were trap-

nested, and the individual eggs collected. Each yolk was separated from the white as completely as possible and gradually diluted with distilled water, 10 ml. of alcohol and 5 ml. of 40 % KOH were added and the whole was made up to 200 ml. Aliquot portions of this were saponified with a further addition of alcoholic potash for 1½ hours in an evacuated pressure-flask on a steam-bath. The saponified solution was then treated according to the method described by Mottram [1910] for the estimation of the fatty acids of liver tissue. The mixed fatty acids were dried to constant weight in an oven through which a current of CO_2 was passing, and the iodine values were determined by Wijs's method. The changes occurring in the egg fat as shown by the iodine values are shown graphically in Fig. 1.

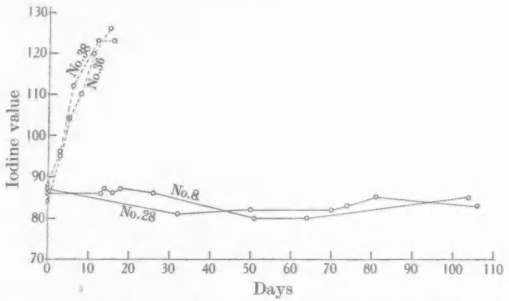

Fig. 1. Effect of ingestion of hemp oil and mutton fat on the mixed fatty acids of egg-yolk.
– – – – Hemp oil (I.V. = 164); ——— Mutton fat (I.V. = 45).

In section (*b*) the experimental rations were as follows:

(1) Fish meal-free mash } Normal rations.
(2) Control mash }
(3) ,, +28 % palm kernel oil.
(4) ,, +28 % palm oil.
(5) ,, +28 % mutton fat.
(6) ,, +28 % linseed oil.
(7) ,, +28 % hemp oil.
(8) Hempseed alone.

The fish meal-free ration (which contained degermed maize, bran, whole yeast, rice, alfalfa and mineral mixture, with 7 % extracted soya bean meal) was fed in addition to the control mash, since it was thought that the fish meal contained in the latter might possibly influence to some extent the composition of the mixed fatty acids of the egg. The ether extract of the former was only 2·3 % as compared with 3·8 % in the control ration, as the constituents were selected for their low fat content. Analyses of the normal rations and of hempseed are given in Table I.

Table I. *Average composition of rations.*

	Moisture %	Ether extract %	Protein %	Carbohydrate %	Fibre %	Ash %
Control	12·5	3·8	14·1	57·9	6·6	5·1
Fish meal-free	10·9	2·3	13·7	67·8	1·3	4·0
Hempseed	8·5	29·5	24·3	15·0	17·9	6·7

Rations 3–8 represent wide differences in the composition of the ingested oils, those in groups 3–5 having a solid acid content ranging from 80 % in the palm kernel oil to 46 % in the palm oil, with unsaturated acids consisting mainly of oleic acid, while in 6, 7 and 8 the percentage of solid acids varies from 6 to 9 %, and the unsaturated acids contain a large proportion of linoleic and a considerable amount of linolenic acid. Analyses of the oils are given in Table II.

Table II.

Composition of oils.

		Hemp oil	Linseed oil	Palm oil	Mutton fat
Oil	I.V.	163·7	180·6	53·1	44·5
	Sap. no.	190·4	191·7	196·0	196·3
	Mol. wt.	294·7	292·6	286·2	285·8
	CNS value	93·1	112·8	—	—
Mixed acids	I.V.	172·9	191·54	55·0	46·1
	Mol. wt.	283·0	282·2	273·8	277·0
	% solid acids	6·1	9·2	46·3	51·0
Liquid acids	I.V.	183·0	206·5	103·8	89·2
	Mol. wt.	280·3	282·0	283·4	276·0
	CNS value	104·7	131·4	—	—
	Percentage composition of mixed fatty acids.				
	Solid acids	6·1	9·2	46·3	51·0
	Oleic acid	11·9	11·41	46·4	46·8
	Linoleic acid	67·2	38·98	7·3	2·2
	Linolenic acid	14·8	40·41	—	—

Palm kernel oil was not examined, but according to Collin and Hilditch [1928] its component fatty acids are: myristic, lauric and lower saturated acids, 70·7 %; palmitic, 8·8 %; stearic, 1·3 %; oleic, 18 %; with only a trace of linoleic acid.

Samples consisting of 10–12 eggs were collected from the different groups after at least 6–8 weeks from the commencement of feeding the experimental rations. The yolks were separated from the whites and ground up with anhydrous sodium sulphate. This material was then extracted in a Bolton and Revis extractor for prolonged periods with anhydrous ether, the solvent being frequently changed, until on evaporation no residue remained. The egg oil thus obtained, which was viscid and orange-coloured, was dried *in vacuo* at 100° and when not analysed immediately was put into small ground stoppered bottles and stored in the dark at −20°.

No attempt was made to separate the neutral fats from the phosphatides, owing to the great difficulty of obtaining either of them in a pure state without admixture with the other. Since the oil obtained by the method just described yielded mixed acids the iodine value of which was only slightly lower than that of the mixed acids obtained by the Mottram saponification method, it was considered that all except very small amounts of the total fatty acids derived from both the neutral fats and the phosphatides had been extracted, and it is probable therefore that the analyses given are representative of the total fatty acids of the egg-yolk. This view is supported by the fact that our results for normal eggs agree well with those of Grossfeld [1933], who, by his method of heating the yolk with hydrochloric acid, undoubtedly obtained the phosphatide-fatty acids as well as the fatty acids of the neutral fat in his mixed acids.

The oil obtained by the extraction method was saponified, the unsaponifiable matter removed, the mixed fatty acids precipitated with hydrochloric acid, taken up in light petroleum and dried *in vacuo* at 100°.

Determinations were made of the iodine value and molecular weight of the mixed acids. The solid and liquid acids were separated by crystallisation of the lead salts from 95 % alcohol [Twitchell, 1921], using the procedure of Hilditch and Priestman [1931]. The iodine value of the solid acids was estimated and the percentage of solid acids corrected for any oleic acid present.

The solid acids always contained traces of a brownish non-fatty material, which did not dissolve during the second precipitation of the lead salts. The molecular weights therefore are probably slightly too high.

The alcoholic solution of the liquid acids was diluted to twice its volume, the liquid acids separated with dilute acetic acid and taken up in light petroleum. The ethereal solution was repeatedly washed till free from acid, the ether evaporated off and the acids dried *in vacuo* at 100°.

The iodine value, molecular weight and thiocyanogen value (CNS value) of the liquid acids were determined. The percentage of hexabromides was estimated in a few cases, but did not prove satisfactory, and it was decided to substitute the thiocyanogen value as a means of estimating the linolenic acid, information having been obtained that this method gave higher and more accurate results [Griffiths and Hilditch, 1934]. Determinations were carried out, with careful attention to the absolute dryness of all reagents and apparatus employed, as recommended by Kaufmann and Keller [1929].

Oleic and linoleic acids were calculated from the iodine value of the mixed acids after allowance had been made for the solid acids and linolenic acid present. It was assumed that acids more unsaturated than linolenic acid were absent, though this was probably not strictly true.

Results of the analyses of the mixed fatty acids of the egg fat are given in Table III.

Table III.

Composition of mixed fatty acids of egg-yolk.

		Control mash	Fish meal-free mash	Mash + palm kernel oil	Mash + palm oil	Mash + mutton fat	Mash + linseed oil	Mash + hemp oil	Hempseed alone
Mixed acids	I.V.	84·4	80·0	80·5	85·9	84·0	123·1	115·7	127·2
	Mol. wt.	280·5	283·0	280·3	281·4	284·0	281·8	282·1	280·6
Solid acids	%	31·4	31·2	30·3	27·8	29·5	23·9	24·3	21·4
	Mol. wt.	263·5	264·0	266·4	265·5	268·0	265·7	267·4	266·0
Liquid acids	I.V.	121·2	115·9	115·0	120·0	117·7	161·3	156·3	162·8
	Mol. wt.	278·2	284·0	278·8	280·0	283·3	283·4	281·1	278·9
	CNS value	94·0	93·3	92·3	90·8	93·7	111·2	96·4	101·8

Percentage composition of mixed fatty acids.

Solid acids	31·4	31·2	30·3	27·8	29·5	23·9	24·3	21·4
Oleic acid	46·7	51·4	51·9	49·8	50·8	33·8	28·8	26·7
Linoleic acid	19·0	15·0	16·1	21·7	16·9	24·9	41·7	41·9
Linolenic acid	2·9	2·4	1·7	0·7	2·8	17·4	5·2	10·0

Discussion.

No effect on the flavour of the egg was observed during the feeding of the experimental rations, except in the case of linseed oil, which produced an unpleasant taste in the soft-boiled egg.

The results obtained on successive eggs of individual hens show that on the hempseed ration the mixed fatty acids became steadily more unsaturated, the normal iodine value of 84–88 being increased to 123–126 at the end of 16 days, and there remaining constant, showing that by this time all the layers of yolk

(which are laid down concentrically) had been deposited under the influence of the hempseed diet. The period required for reaching the maximum iodine value is a measure of the period required for the growth of the ovum. This is not necessarily a constant, but will differ in different individuals, or even in the same individual at different seasons, according to the rate of production.

Compared with hempseed, mutton fat had little effect on the iodine value of the mixed acids. A slightly lower value was obtained from the 7th to the 10th week after the commencement of the period, but towards the end it returned to the normal.

It appears therefore that while unsaturated oils given in the form of a diet such as hempseed readily affect the composition of the yolk fat, as indicated by the change in iodine value, the ingestion of a hard fat, *viz.* mutton fat, in conjunction with a cereal ration, does not significantly affect it, even after prolonged feeding.

Reference to Table III shows that on a cereal diet with a protein supplement, the degree of saturation of the mixed acids of the egg is similar to that of the mixed acids of the body fat on a normal ration. Solid acids constitute about 31 %, and according to the molecular weight (263·7) appear to consist mainly of palmitic acid (molecular weight 256).

Oleic acid is the main constituent of the liquid fraction, with smaller amounts of linoleic and 2–3 % of linolenic acid, as determined by the thiocyanogen value. The percentages of linoleic (15–19 %) and of oleic acid (47–51 %) are of the same order as those found by Grossfeld [1931] in hen depot fat. The composition of the mixed fatty acids from the control and fish meal-free eggs is very similar to that recorded by Grossfeld [1933] for commercial eggs of Danish origin.

Since the fish meal-free ration contained only 2–3 % of ether extract, it is evident that a considerable proportion of the normal egg fat must be derived by synthesis from the non-fatty constituents of the food rather than by direct assimilation of fat. It cannot be definitely stated whether synthesis of linolenic acid from carbohydrate takes place or not, since both the control and fish meal-free rations contained soya bean meal, which, even when solvent-extracted, may contain traces of this acid.

The addition of palm kernel oil to the ration produced a fatty acid mixture of the same composition as that of the fish meal-free eggs. No lowering of the molecular weight of the mixed acids or of the iodine value of the liquid acids was observed, as would have been the case had appreciable amounts of the lower saturated acids been deposited.

The ingestion of palm oil and mutton fat did not affect the composition to any significant extent, though in the former case the percentage of linolenic and of solid acids was slightly reduced, and that of the linoleic acid slightly increased.

With the feeding of unsaturated oils a marked increase in the degree of unsaturation took place, accompanied by a decrease in the percentage of solid acids, a marked decrease in oleic acid and an increase in both linoleic and linolenic acids.

The iodine value of the mixed acids was highest, and the percentage of solid acids lowest, on the hempseed diet, *i.e.* where the ration fed, though rich in unsaturated fatty acids and protein, was relatively deficient in carbohydrate. When the hemp oil was fed in conjunction with a carbohydrate-rich ration, the degree of unsaturation was reduced and the percentage of solid acids slightly raised, as compared with the hempseed diet. The mash *plus* linseed oil produced a greater degree of unsaturation in the mixed acids than the mash *plus* hemp oil,

in accordance with the iodine values of the respective oils. The percentage of solid acids was the same in both cases.

The percentage of linoleic acid appears to be related to some extent to that of the ingested oils. The linoleic acid content of the mixed acids of the oils, and of the mixed acids of the eggs produced on the rations containing them, are as follows: hemp oil, 67 %; hempseed and hemp oil eggs, 42 %; linseed oil, 39 %; linseed eggs, 25 %; mutton fat and palm oil, 2–7 %; mutton fat and palm oil eggs, 17–22 %. Even when no added linoleic acid is present in the diet the linoleic content of the egg fatty acids will be approximately 15–19 %.

The linolenic acid content also tends to run parallel with that of the ingested oils, the largest amount being found when linseed oil is fed, the percentage here being over five times that found in the normal egg. Hempseed fed alone raised the percentage of linolenic acid in the mixed acids to three times that of the normal egg. In this case the linolenic acid content of the liquid acids, *viz.* 12·7 %, approximates to that present in the oil itself.

When the diet contained ample carbohydrate in addition to the hemp oil, however, the linolenic acid was reduced by 50 %. To what extent the linolenic acid content might have been raised on linseed alone could not be determined, as, owing to its unpalatability, fowls can rarely be induced to eat more than small amounts of the seed.

From these results it appears that the hen, even when abundant carbohydrate is present for the synthesis of normal fat, will utilise ingested fatty acids for direct deposition in the egg fat. When the ration containing the unsaturated fatty acids is deficient in carbohydrate, a greater proportion of the acids will be deposited. On the other hand, in spite of the ingestion of large amounts of saturated acids in the presence of ample carbohydrate, the composition of the mixed fatty acids of the egg tends to remain similar to that produced on a cereal *plus* protein ration containing 2–4 % of ether extract.

It seems possible that in the synthesis of egg fat, the hen exercises a selective influence, and there may be a degree of saturation of the fat which cannot be increased by the ingestion of saturated fatty acids, but which can be reduced to a marked extent by the assimilation of unsaturated acids.

Since the fat of the egg-yolk serves as a source of energy for the developing embryo, the fact that its composition can be materially influenced by certain nutritional factors may be of considerable significance in connection with embryonic nutrition, hatchability, *etc.*

SECTION II.

Effect of ingested fat on the composition of the depot fat.

The dependence of the constitution of the fat reserves on the composition of the diet has been clearly established in the case of the pig, notably by Ellis and co-workers [1926; 1930; 1931] in their extensive studies on soft pork, and by Bhattacharya and Hilditch [1931], and in the case of the rat by Anderson and Mendel [1928], Eckstein [1929], Reed *et al.* [1930], Banks *et al.* [1933] and others. All these investigators have found that dietary fat may exert a marked effect on the composition of the depot fat.

Little attention however has been directed towards the study of fat formation in birds. Quantitative data exist for the composition of goose fat [Bömer and

Merten, 1922], and for the fat of the hen [Grossfeld, 1931], but the effect of diet on such composition has received little consideration.

Henriques and Hansen [1903] found that the iodine values of the fat of three hens fed on a diet of hempseed were 103, 120 and 135 respectively. Schirmer [1921] records an iodine value of 74·8 for the fat of geese on a diet of maize and olive oil, and 63·2 on maize and mutton fat. In spite of the statement in a report from the Kansas Agricultural Experiment Station [1922] that "none of the short chain fatty acids of the food are deposited in the body fat of the hen, while the unsaturated fatty acids are so deposited" the actual tabulated data show clearly that either saturated or unsaturated fats can be deposited in the fat depots of the hen.

Experimental.

The birds in this section of the experiment were kept under the conditions of management already described. The same control ration was employed.

The fats investigated, *viz.* palm kernel oil, mutton fat and hemp oil, provided examples of fats differing considerably in composition from that of the normal body fat. Since the inclusion of small amounts of fat in the ration only slightly affected the normal composition of the body fat in the pig, it was decided to incorporate the palm kernel oil and mutton fat in the control ration at a high level, *viz.* 28 %. In investigating the effect of hemp oil, hempseed was fed as the sole diet, owing to the difficulty of obtaining sufficient quantities of the oil

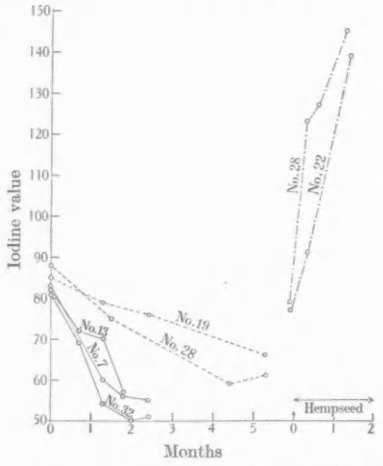

Fig. 2. Effect of ingestion of palm kernel oil, mutton fat and hemp oil on the mixed fatty acids of abdominal fat.

——— Palm kernel oil (I.V. = 15); – – – – Mutton fat (I.V. = 45);
•—•—• Hemp oil (I.V. = 164).

itself. The percentages of fat in these three diets were very similar, since the hempseed contained 28–29 % of ether extract. It was however richer in protein and considerably poorer in carbohydrate than the fat *plus* mash rations.

The work of Scott [1920] having shown that considerable individual variation in the composition of the body fat occurs in pigs even under the same

conditions of management and dietary régime, it was decided to study progressive samples of body fat obtained from the same individual during the experimental period.

Samples of fat (0·5–0·8 g.) could be readily removed without injury to the bird from the abdominal region, where there is a comparatively large superficial deposit in a well-nourished hen. These were taken while the birds were still on the control ration, and again at intervals during the experimental period. The fat was saponified in evacuated pressure-flasks and the iodine value determined on the mixed fatty acids. The results are shown graphically in Fig. 2.

In pigs and cattle it has also been found that the composition of the fat varies significantly in different areas, therefore samples from the various fat depots were examined to ascertain whether such variation in composition occurs in the fowl. The results are shown in Tables IV and V. As the iodine values of the different areas were very similar, it was assumed that the abdominal layer could safely be taken as representative of the body fat as a whole.

Table IV. *Iodine values of mixed fatty acids of fat from different depots.*

No. of hen	Gizzard	Neck	Leg muscle	Abdominal
3	93	92	93	90
15	79	79	—	78
17	64	67	65	65
25	90	90	90	88

Table V. *Iodine values of mixed fatty acids of fat from different depots.*

No. of hen	Skin	Neck	Leg muscle	Abdominal	Gizzard	Kidney	Mesenteric	Epicardial	Av. I.V.	Max. diff. between depots
5	87·3	88·1	86·2	87·1	84·5	84·4	84·0	85·6	85·9	4·1
8	81·4	78·0	—	—	75·6	77·7	—	—	78·2	5·8
29	92·0	89·98	88·4	88·1	89·0	87·8	88·0	90·2	89·2	4·2
32	85·9	81·7	81·2	80·8	79·7	81·6	80·7	85·7	82·2	6·2

Discussion.

Composition of different fat depots.

In the hen fat is deposited as a thick abdominal layer and as a layer surrounding the gizzard. Smaller deposits are found in the neck region, chiefly between the clavicles, and also along the ventral muscles of the femur. The last-mentioned two deposits are relatively superficial.

Table IV shows that the iodine values of the fatty acids of the main fat depots are very similar. Since this is in contrast to the findings in pigs and cattle, examination of the smaller depots was made, with the results shown in Table V. The skin fat was obtained by extraction of the skin itself, which contains a certain amount of fat along the tracts of the feather follicles. The gizzard fat was obtained from the dorsal side of the gizzard only, as the fat on the ventral side is more or less continuous with the abdominal layer. The differences in iodine value are slightly greater than in Table IV but are nevertheless much smaller than those recorded for pigs and cattle. In two cases the skin is the most unsaturated layer, in one the epicardial fat is equally high, in the fourth the neck fat is one unit higher. It is evident that the most deep-seated layers, *viz.* kidney, mesenteric and epicardial fat, do not show a decidedly lower iodine value than the subcutaneous fat, as recorded by Henriques and

Hansen [1901] in cattle, where the iodine value of the skin fat was 52, and that of the mesenteric fat 40. Even greater differences were found by Bhattacharya and Hilditch [1931] in pigs on a normal ration, where the iodine value ranged from 63 in the outer back fat to 46 in the perinephric fat. The various fat depots of the hen therefore, judged by the iodine values, appear to be of more uniform composition than is the case in certain mammals. That the uniformity extends also to the component fatty acids has been shown by the detailed analyses of Hilditch *et al.* [1934]. This fact lends support to the suggestion that the temperature of the different areas is not the sole factor in determining the composition of the fat reserves [Dean and Hilditch, 1933].

The variation between individuals in Table IV is due to the fact that the birds were taken from the fat-fed groups at various times. No. 8 (Table V) had also received a ration containing mutton fat for 6 weeks. Nos. 5, 29 and 32, however, which were all on the control ration, show that some individual variation in composition exists, even under identical nutrition and management, as is the case also in pigs [Scott, 1920].

Effect of ingested fat on the composition of the body fat.

In Fig. 2 individual variation is again evident in the five hens examined at the commencement of the experimental period, while they were still on the control ration, the iodine values ranging from 81 to 88. A steady change towards a harder fat occurred when rations containing 28 % of mutton fat and of palm kernel oil were fed. Individuals however varied slightly in rate and degree of response to the diets. For example, in the case of No. 19 the mutton fat ration lowered the iodine value from its original level (85) to 66 at the end of about 5 months, while in No. 28 the iodine value fell from 88 to 59 in $4\frac{1}{2}$ months. After this the iodine value of No. 28 appeared to continue at practically the same level. Three other hens (not shown in the figure) on the mutton fat mash were operated on, and it was evident from observation of the birds that the individual response to the feeding depended in a large measure on the physiological condition of the bird (whether laying, non-laying or broody) and the resulting capacity for food consumption. The iodine value in No. 28, whose food intake was very high, fell lower than in any of the other birds examined.

The birds on the palm kernel oil mash showed a marked response to the ingestion of this oil, the iodine value falling from the original level (81–83) to 51–55 in a little over 2 months. The more rapid change in this case was probably due to two causes. (1) The iodine value of the palm kernel oil, *viz.* 15, is much farther removed from that of the body fat than is that of mutton fat, *viz.* 45, hence the ingestion of equal amounts of the two fats would result in a greater decrease in iodine value in the case of the palm kernel oil feeding. (2) The palm kernel oil mash was obviously much more palatable to the birds than the mutton fat mash, and food consumption was therefore greater.

Four birds in the hempseed group were operated on. In each case a rapid and pronounced change in the direction of increased unsaturation took place. The results for two of the birds are shown in Fig. 2. No. 28 showed the most rapid and most marked response, the iodine value of 79 at the commencement of the period being raised to 145 within 6 weeks. In No. 22 the iodine value rose to 139, and in the other two birds to 124 and 130 respectively within the same period.

With regard to these data it should be noted that though the fat content of the diet was approximately the same in the three groups, the birds on hemp-

seed were receiving a ration which was considerably richer in protein but contained only a third of the carbohydrate content of the fat *plus* mash rations. In consequence, they probably tended to lay down the ingested fat more rapidly than if supplied with a ration containing an adequate amount of carbohydrate from which the normal body fat could be synthesised. The results show clearly, as did the tabulated data of the Kansas Agricultural Experiment Station Report [1922] that the degree of saturation of the mixed fatty acids of the body fat can be either increased or decreased by the ingestion of appropriate fats in the ration.

The record of the variations in the iodine value of the mixed fatty acids of the abdominal fat of No. 28 over the 12-month period during which she was under experiment seems worthy of note (see Table VI).

Table VI. *Changes in iodine value of mixed fatty acids of abdominal fat of No. 28.*

Date	I.V.		Date	I.V.	
—	88	Control ration	10. xii. 31	123	Hempseed ration
1. vi. 31	—	Mutton fat ration	17. xii. 31	127	,,
14. vii. 31	75	,,	9. i. 32	145	,,
8. x. 31	59	,,	16. i. 32	—	Control ration
10. xi. 31	61	,,	28. i. 32	139	,,
—	—	Control ration	10. ii. 32	137	,,
30. xi. 31	79	—	3. iii. 32	115	,,
1. xii. 31	—	Hempseed ration	2. vi. 32	90	,,

It will be seen that saturation has taken place more slowly than unsaturation, both during the production of a more saturated fat than the normal, and in the production of a normal fat from an abnormally unsaturated one. This does not necessarily mean that the hen tends to select the unsaturated acids more readily than the saturated acids, since various factors may have combined to produce this effect. The palatability of the respective rations, the ratio of fat to carbohydrate present, the degree of divergence of the iodine value of the ingested fat from that of the body fat, the varying appetite and condition of the bird, must all be taken into account.

The rapidity with which the experimental rations have influenced the composition of the body fat is not very surprising in view of the hen's intense physiological activity. In the fowl, the weight of thyroid per kg. of body weight, and also the percentage of iodine in the thyroid are greater than in mammals [Cruickshank, 1929]. A higher metabolic rate therefore results, as shown by the body temperature (104–108° F. as compared with 101° F. in the cow, or 103° F. in the pig). Respiration and heart beat, digestion and absorption are more rapid, while food consumption per unit of body weight is considerably greater. The high metabolic rate is essential in a system of reproduction entailing a rapid transformation of raw materials into food constituents for embryonic use. In a hen laying 250 eggs *per annum*, this involves an output of 31 lbs. of eggs, of which 4·1 lbs. are protein, 3·3 lbs. are fat and 3·1 lbs. are $CaCO_3$ in the form of shell.

From our results it appears that the selection which the hen exercises with regard to the deposition of ingested fat in the egg does not hold in the case of the body fat, which, so far as can be ascertained by the iodine value, may be markedly influenced by the fatty acids ingested, irrespective of their degree of saturation. It seems possible also that changes in the composition of the fat can be more rapidly induced in the bird than in the mammal, owing to the higher rate of metabolism of the former and the resulting rapid utilisation of food.

SUMMARY.

Effect of ingested fat on the composition of the egg fat. On normal cereal rations containing protein supplements, the mixed fatty acids of the egg fat contained about 31 % solid acids, 47–51 % oleic acid, 15–19 % linoleic acid and 2–3 % linolenic acid.

While the degree of saturation and the proportion of the component fatty acids could be considerably modified by the ingestion of unsaturated fatty acids, the ingestion of saturated acids had relatively little effect in altering the normal composition of the mixed fatty acids of the egg fat.

Effect of ingested fat on the composition of the depot fat. The iodine values of the mixed fatty acids of the different fat depots in the hen were determined. These indicated that the superficial and internal fat reserves are more uniform in composition than in the case of pigs and cattle.

Observations on individual birds showed that the ingestion of high percentages of saturated fatty acids in the form of palm kernel oil and mutton fat definitely decreased the degree of saturation of the mixed fatty acids of the depot fat, while the ingestion of unsaturated acids in the form of hempseed resulted in a marked and rapid increase in unsaturation.

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CXXXVII. BILE PIGMENTS. VI. BILIVERDIN, UTEROVERDIN AND OOCYAN.

By RUDOLF LEMBERG.

From the Biochemical Laboratory, Cambridge.

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By dehydrogenation of bilirubin with ferric chloride a green pigment, dehydrobilirubin, has been prepared. The same pigment is found in nature as oocyan in the egg-shells of gulls and other birds [Lemberg, 1931] and as uteroverdin in the dog's placenta [Lemberg and Barcroft, 1932]. Dehydromesobilirubin, which differs from dehydrobilirubin merely in having saturated side-chains, was prepared by Fischer *et al.* [1932] and called glaucobilin.

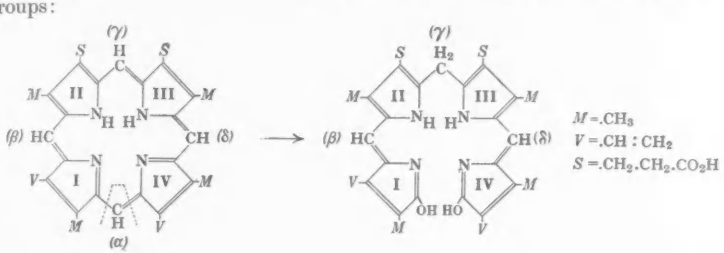
Analysis of the dimethyl esters of these pigments showed that these compounds possess two hydrogen atoms less than bilirubin and mesobilirubin, but the same number of oxygen atoms, *i.e.* six. The first analytical values of the oocyan ester showed some divergence, but later analyses [Lemberg, 1932] gave the same results for oocyan and dehydrobilirubin esters. The constitution of dehydrobilirubin is established in the present paper by analyses of dehydrobilirubin (free acid). The first analyses of this substance favoured a formula with eight oxygen atoms, while the results of Fischer on glaucobilin (free acid) corresponded to a formula with seven oxygen atoms. Analyses of carefully purified dehydrobilirubin establish the formula $C_{33}H_{34}O_6N_4$. The analyses of the free acid and the esters show furthermore that only two hydrogen atoms have been removed by the dehydrogenation of bilirubin.

A second point which required further investigation was the relation of dehydrobilirubin and dehydromesobilirubin to biliverdin and mesobiliverdin, pigments showing similar colours and absorption spectra, which are obtained by autoxidation of bilirubin and mesobilirubin in alkaline solution. It is now shown that "mesobiliverdin" is actually a mixture consisting largely of dehydromesobilirubin together with certain by-products which cannot be obtained in a pure state, but give the impression of being resinified substances. The yield of pure dehydrobilirubin from "biliverdin" is lower. This is to be expected as the unsaturated side-chains form a point of attack for alkali and oxygen. It seems preferable now to apply the convenient name "biliverdin" to pure crystalline dehydrobilirubin, instead of to a mixture of the latter with products of secondary alteration. The name mesobiliverdin rather than dehydromesobilirubin or glaucobilin will in future be used for the product with saturated side-chains.

A further simplification is rendered possible by the evidence now brought forward that the differences in optical properties between crystals of uteroverdin ester and oocyan ester, observed by Bernal in 1931 [Lemberg and Barcroft, 1932], are due merely to differences in growth and formation of the crystals, and probably do not indicate structural difference. Further investigation has confirmed the existence of two different crystal forms of biliverdin ester differing only with regard to pleochroism and extinction. One of these forms is obtained from biliverdin synthesised from pure bilirubin, the other (usually mixed with

the first) from biliverdin obtained from bilirubin mother-liquors and from the natural biliverdins, particularly uteroverdin.

This matter has been carefully investigated, as the existence of slight differences between isomeric bile pigments might have an important and interesting significance. According to recent investigations of Fischer and his collaborators bilirubin is represented by a formula in which four pyrrole nuclei are linked together by three carbon atoms to form an open chain. Bilirubin arises from protoporphyrin by oxidative opening of the porphyrin nucleus; one of the four CH-groups of the ring (α in the formula) is removed and replaced by two hydroxyl groups:



Fischer and Hess [1931] had assumed a symmetrical position of the methyl and vinyl groups in the rings I and IV of bilirubin (CH_3 next to OH in ring IV, instead of vinyl as in the formula above). This would have meant that the formation of bilirubin from protoporphyrin implies decomposition of the latter and resynthesis, as the position of the side-chains in ring IV could not be changed in any other way. Later, however, Siedel and Fischer [1933] proved that the side-chains of bilirubin are unsymmetrically arranged exactly like those in haemin and protoporphyrin. The ring is opened between the rings I and IV and the α-CH-group is removed.

Now three other bilirubins may arise from protoporphyrin if the ring could be opened at one of the other CH-groups (β, γ, δ) instead of at α. Thus isomeric bilirubins would originate differing from each other in the position of the side-chains attached to the rings. Such isomerides are often extremely similar and may even give no depression of the melting-point if mixed. Examples are found in the paper of Siedel and Fischer [1933] and previous ones of Fischer, *e.g.* the three mesobilirubins (IIIα, IXα, XIIIα) differing in the position of the side-chains attached to the rings I and IV. The difference of the two forms of biliverdin ester, if actually due to a structural difference, could only be caused by a difference in the position of the side-chains. The absence of a melting-point depression, the analytical data, the identity of the absorption curves and of other properties (such as basicity and solubility) leave no doubt that the nuclear system of the four pyrrole rings and the arrangement of the double bonds are the same in both forms.

The biliverdin ester of the form predominantly present in uteroverdin (which is different from the form obtained from pure bilirubin) can be obtained from mother-liquors of bilirubin and from gallstones. If thus structurally different types of biliverdin exist, then it is almost certain that there are two corresponding structurally different bilirubins. Uteroverdin, moreover, is formed in haemorrhages in placental tissue and is definitely of extrahepatic origin; thus the different biological origin is possibly connected with the chemical difference.

In the crystallographic investigations of the esters and their hydrochlorides I had the valuable help of Mr Rawlins of the Mineralogical Laboratory at Cambridge. These investigations show that the assumption of a structural difference between the two different crystalline forms is unnecessary, and that uteroverdin, oocyan and biliverdin may be considered identical. These investigations do not necessarily exclude the above explanation of the observed differences, but they give no evidence for it. Two different forms of protoporphyrin crystals have been observed by Richter [1930], one of which is caused by impurities according to Fischer *et al.* [1931] and Hamsik [1931][1].

The microanalytical part of the work has been done by Dr Roth, Heidelberg.

Experimental.

Mesobiliverdin by autoxidation of mesobilirubin.

The autoxidation of mesobilirubin to mesobiliverdin in alkaline solution was studied under different conditions and the product of the reaction was isolated. The effect of piperidine was tried, as this substance accelerates the autoxidation of dihydropolyenes [Kuhn and Drumm, 1932].

Five small crystallising dishes were filled as follows: I, 1·7 ml. 0·22 *N* NaOH. II, 3 ml. of the NaOH and 1 ml. water. III, IV and V like II but with the addition of 0·1 ml. *M*/10 ferrous sulphate solution to III, 0·01 ml. of the same solution to IV and one drop of piperidine to V. In each of them 0·1 g. of mesobilirubin was dissolved requiring theoretically 1·54 ml. of the NaOH solution (2 mol.). I contained 2·2, II–V 3·9 mols. of alkali. The dishes were placed in a water-filled desiccator and kept in it at 30°. After 12 hours only the solution in IV had turned green, the other ones were olive-brown. After 4 days' standing I and IV were green; II, III and V olive-green. Therefore the reaction proceeds best if a great excess of alkali is avoided. The reaction is accelerated by traces of iron, but not by piperidine.

The solutions I and IV were mixed and acidified with dilute acetic acid. The pigment (from 0·2 g. mesobilirubin) was extracted with ether, and after evaporation of the ether, the residue was extracted with methanol, which left some unchanged mesobilirubin undissolved. The mesobiliverdin was then esterified by passing in hydrogen chloride. After keeping overnight, esterification was completed by boiling the solution, diluted previously with half its volume of absolute methanol, for 15 minutes. It was then evaporated *in vacuo*. The residue was dissolved in a small amount of methanol, sodium acetate solution was added and the ester was extracted with ether. The extract was washed with 1 % sodium carbonate solution and water. The ether was evaporated and the product recrystallised from methanol. 31 mg. of pure mesobiliverdin dimethyl ester crystallised out. M.P. 221° (corr.). It was identical in every respect with glaucobilin ester [Fischer *et al.*, 1932].

The same substance was obtained from II, III and V, but the yield was smaller.

Biliverdin by autoxidation of bilirubin.

0·58 g. of bilirubin was dissolved in 10·5 ml. of 0·22 *N* NaOH and 0·07 ml. of *M*/10 ferrous sulphate solution was added. After 6 days' standing at 30° some precipitated bilirubin (10 mg.) was filtered off and the biliverdin taken up in ether and precipitated therefrom as described below. 150 mg. of crude biliverdin were obtained, which however gave only 10 mg. of well crystallised pure biliverdin

[1] Lindenfeld [1931; 1933] considers the different crystalline forms of haemin and porphyrin esters to be polymorphous modifications.

dimethyl ester. M.P. 220–221° (corr.). The crystals were those termed form A below. No depression of the melting-point was obtained with dehydrobilirubin dimethyl ester [Lemberg, 1932].

The considerable ether-insoluble part of the green pigment was almost insoluble in methanol. From its solution in pyridine it was precipitated by hot methanol in very fine amorphous flocks of almost black colour and resinous character. Dilute methyl alcoholic hydrochloric acid dissolved it with an olive-brown colour due to included bilirubin; more concentrated methyl alcoholic hydrochloric acid again precipitated black flocks.

Mesobiliverdin from the products of the Gmelin test for mesobilirubin.

0·1 g. mesobilirubin was dissolved in 100 ml. of warm chloroform. A few ml. of chloroform were shaken in a test-tube with one drop of fuming nitric acid, and 1 ml. of this added to the mesobilirubin solution. The colour changed slowly to olive-green. After 6 minutes the colour had become green with a yellow tinge at the borders of the liquid. The reaction was stopped at this time by shaking with water containing a little sodium acetate. The chloroform was evaporated and the residue taken up with absolute methanol which left 30 mg. of unchanged mesobilirubin undissolved. The solution was blackish-blue in neutral, blue in acid solution. The acid solution showed, besides the absorption in the red due to mesobiliverdin, a weak band at 590 $m\mu$. On addition of zinc acetate the neutral solution turned green with weak red fluorescence and showed three bands, the strongest at 685 $m\mu$, a weaker one at 630 $m\mu$ and a very weak one at 578 $m\mu$. The violet pigment which causes the band 590 $m\mu$ in acid solution and the bands at 630 and 578 $m\mu$ and the red fluorescence of the zinc salt will be discussed in another paper.

The pigment in the methyl alcoholic solution was esterified and the ester brought into ethereal solution as described above. From the ethereal solution mesobiliverdin ester was extracted with 1 % hydrochloric acid which showed now the characteristic blue-green colour and absorption only in the far red. The by-products, among them the weakly basic violet pigment, remained in the ether. The mesobiliverdin ester was shaken out with fresh ether after addition of dilute sodium carbonate solution. It was recrystallised from methanol. 25 mg. of steel-blue prisms of mesobiliverdin dimethyl ester were obtained. M.P. 220° (uncorr.).

If the Gmelin reaction were allowed to proceed until the blue phase was reached the yield of mesobiliverdin became very small.

Mesobiliverdin by oxidation of mesobilirubinogen with ferric chloride.

0·3 g. mesobilirubinogen (the chromogen of mesobilirubin) was boiled for 1·5 hours with a solution of 1·5 g. $FeCl_3$ in 9 ml. 25 % hydrochloric acid and 50 ml. methanol. On cooling well formed rectangular blue leaflets with straight ends crystallised out. Yield 0·2 g.

Although the crystals looked perfectly uniform, they were mixed crystals of two different substances. Their blue solution in acid alcohol showed, besides strong absorption in the red, a band at 595 $m\mu$ and one at 555 $m\mu$ with a shading between the two. In the neutralised solution on addition of zinc acetate a strong red fluorescence appeared and the blue-green solution showed bands at 685, 631 and 578 $m\mu$. If the crystals were recrystallised twice from methanol containing some HCl flat blue-green needles with straight ends were obtained, whereas the mother-liquor was blue. The solution of the crystals now showed absorption only in the red; the zinc compound was green and without fluorescence. The

mother-liquors gave a blue zinc compound with strong red fluorescence and the bands 631 and 578 $m\mu$; the acid solution showed the bands at 595 and 555 $m\mu$. These bands are caused by mesobiliviolin. The crystals are the ferric chloride double salt of mesobiliverdin dimethyl ester (the "ferrobilin ester" of Fischer *et al.* [1932]). M.P. 276° (not corr.). (Found: C, 52·04; H, 5·34; Cl, 16·81; Fe, 5·72; OCH_3, 7·52 %. $C_{35}H_{42}O_6N_4.HCl.FeCl_3$ requires C, 51·66; H, 5·33; Cl, 17·45; Fe, 6·87; $2OCH_3$, 7·63 %.) The low value for iron is explained by the fact that $FeCl_3$ is volatile and iron was assayed as combustion residue.

Optical properties of the crystals: pleochroism: blue, if long axis of platelets parallel to plane of polarisation (NS), blue-green in perpendicular orientation (EW)[1]. Extinction almost straight.

By mild treatment with alkali they were transformed into mesobiliverdin ester as described by Fischer *et al.* [1932].

Similar mixed crystals to those mentioned above, but richer in mesobiliviolin chloride, result from shorter treatment of mesobilirubinogen with ferric chloride. They have been obtained previously and considered to be pure mesobilicyanin chloride [Lemberg, 1933]. It will be shown in another paper that "bilicyanins" are mixtures of pigments, which are coloured blue-violet in acid, red-violet in neutral solution, with biliverdins. For this reason the term violin is used, although the "mesobiliviolin" of Fischer and Niemann [1924] is a mixture of still more complicated nature.

Preparation and analysis of biliverdin.

Free biliverdin (dehydrobilirubin) is rather insoluble in ether. The yield could be greatly increased and much ether saved by using for extraction ether rich in methyl alcohol.

The crude ferric chloride double salt of biliverdin chloride was prepared from bilirubin by oxidation with ferric chloride in glacial acetic acid as described by Lemberg [1932]. 0·5 g. of this material is dissolved in 30 ml. of $N/5$ NaOH and filtered from ferric hydroxide; 100 ml. of methanol are added, the solution is acidified with dilute acetic acid and immediately poured into 500 ml. ether and shaken. It is then extracted several times with ether, some more methanol being added after each extraction. The solution must be kept acid with acetic acid. The extraction is continued until ether no longer extracts an appreciable amount of the pigment, even if some more methanol has been added. 1·5 l. of ether have been found necessary. The remaining watery solution contains a fine precipitate of ether-insoluble "biliverdin" which is filtered off and dried (80 mg. of this by-product).

The ether solution is washed with water. The first washings remove with the methanol a part of the pigment. This is precipitated by addition of some more sodium acetate (the reaction must remain slightly acid) and filtered off or taken back into ether. The greater part of the biliverdin precipitates from the ether solution when the methanol is washed away with water. If the ethereal filtrate is concentrated to 50 ml. and washed again, a second portion of the pigment is obtained. The ether remains violet and contains some biliviolin.

The green precipitates of the ether-soluble part are collected, washed carefully and dried in the desiccator. They are dissolved by boiling with 200 ml. of methanol. The solution is filtered and concentrated to 30 ml.; 240 mg. of

[1] The phrases North-South (NS) and East-West (EW) refer to the position in the field of view of the polarising microscope.

biliverdin crystallise out. This yield, however, can be obtained from the first crystallisation of the ferric chloride double salt only. A much smaller yield is obtained and more of the amorphous ether-insoluble by-product, if that part of the ferric chloride compound is used which comes out of the glacial acetic acid mother-liquors. Thus the total yield of crystalline product is no more than 30 % of bilirubin.

The substance was dried for analysis at 80° and 0·1 mm. over phosphorus pentoxide. (Found: C, 64·63, 64·89; H, 6·00, 5·95; N, 9·06, 8·91, 9·07 %. $C_{33}H_{34}O_6N_4$ requires: C, 68·01; H, 5·89; N, 9·62. $C_{33}H_{38}O_8N_4$ requires: C, 64·04; H, 6·19; N, 9·06 %.)

The analysis of the dimethyl ester of this preparation showed that it contains six oxygen atoms like that of Lemberg [1932]. The starting material, the crude ferric chloride double salt also contains six oxygen atoms only. (Found: C, 50·73; H, 4·59; N, 6·75, 6·67, 7·04; Cl, 17·54, 17·34; Fe, 6·42, 7·11 %. $C_{33}H_{34}O_6N_4.HCl.FeCl_3$ requires: C, 50·71; H, 4·52; N, 7·17; Cl, 18·18; Fe, 7·16 %.) The first iron value was calculated from the weight of the combustion residue, the second obtained by direct precipitation of ferric hydroxide with ammonia. After the biliverdin had been recrystallised twice from methanol the analysis now confirmed the formula with six oxygen atoms for the free acid as well. (Found: C, 67·87; H, 5·79 %. $C_{33}H_{34}O_6N_4$ requires: C, 68·01; H, 5·89 %.) The analytical values for H show that biliverdin has no more than two hydrogen atoms less than bilirubin. (For the free acid have been found: H, 6·00, 5·95, 5·79 %. $C_{33}H_{34}O_6N_4$ requires: H, 5·89 %; $C_{33}H_{32}O_6N_4$: H, 5·56 %. Dimethyl ester, found: H, 6·42, 6·50, 5·96 % (uteroverdin ester); 6·26 % (oocyan ester); 6·39, 6·44 % (biliverdin ester). $C_{35}H_{38}O_6N_4$ requires: H, 6·28 %; $C_{35}H_{36}O_6N_4$: 5·96 %.)

The ether-insoluble by-product still contains some biliverdin. From its methyl alcoholic solution, methyl alcoholic HCl gives a fine blackish precipitate. If this is filtered off, a mixture of amorphous and crystalline chlorides is obtained by addition of hot 2*N* HCl.

Chlorides of biliverdins.

Mesobiliverdin chloride. 50 mg. of mesobiliverdin are dissolved in 15 ml. of boiling methanol and 40 ml. of hot 2*N* aqueous hydrochloric acid are added. After a short time an amorphous precipitate appears; this is filtered off. The chloride crystallises from the filtrate in fine green needles.

The substance is dried for analysis *in vacuo* at 20° over P_2O_5 to avoid dissociation and loss of HCl. (Found: N, 8·78, 8·98; Cl, 4·46, 4·21 %. $C_{33}H_{38}O_6N_4.HCl$ requires: N, 8·98; Cl, 5·70 %.)

Mesobiliverdin dimethyl ester chloride is obtained in the same way. It crystallises also, on standing, from its solutions in 1 % hydrochloric acid obtained by extracting its ether solution with the former: M.P. 175–176°; no complete melting, but sintering together.

Biliverdin chloride. Fine green needles. Extinction slightly oblique. Pleochroism: (NS) blue-green (darker), (EW) yellowish-green (lighter). (Found: C, 63·38, 63·36; H, 5·52, 5·75; N, 7·02, 6·84; Cl, 4·81, 4·71 %. $C_{33}H_{34}O_6N_4.HCl$ requires: C, 63·99; H, 5·55; N, 9·05; Cl, 5·74 %.) The nitrogen is 2 % too low. On drying at 0·1 mm. and 80° over phosphorus pentoxide for several hours, 9·615 mg. substance lost 0·363 mg., but there was no considerable increase in nitrogen percentage. (Found: N, 7·09 %.) There can be no doubt however about the existence of a monochloride.

Biliverdin dimethylester chloride. Fine green needles with no definite M.P. The crystal properties are given below.

Complex salts of biliverdins.

For these experiments mesobiliverdin ester was used.

Zinc salt. A few ml. of a solution of the ester in methanol were filled into a Thunberg tube, in which was placed a small test-tube containing zinc acetate solution. The tube was evacuated twice and filled with nitrogen free from oxygen. When the solutions were mixed the colour turned immediately from blue to green. The general absorption in the red part of the spectrum gave place to a sharper band, with its centre at 685 $m\mu$. This is the band of the complex zinc salt. There is however no fluorescence.

On addition of water and ether, the zinc compound passed from methanol into the ethereal layer. When this was shaken with $N/10$ HCl, the zinc compound was decomposed; the liberated mesobiliverdin ester passed into the acid, showing the typical green colour.

On standing exposed to the air the zinc compound was slowly changed, much faster if ammonia is added. The red fluorescence and the absorption band at 630 $m\mu$ characteristic of biliviolins appeared.

Copper salt. The formation of the copper salt was carried out in nitrogen as in the case of the Zn salt. The solution turned olive-yellow when the copper acetate solution was added. No distinct band could be seen, but general absorption in the red, blue and violet. When the copper compound was brought into ether and this shaken with $N/10$ HCl, no mesobiliverdin was extracted. The ethereal solution was now green and showed a strong band at 650 $m\mu$. When now $2N$ HCl was added, a play of colours developed. Immediately under the green ethereal layer the acid aqueous layer showed a blue zone with a red-violet one below. Gradually the acid solution turned blue-violet, but after some time green flocks of mesobiliverdin chloride precipitated and left the solution red-violet, showing absorption bands at 600 and 550 $m\mu$.

There must exist a copper compound, or otherwise the mesobiliverdin would have been extracted by the dilute acid from ether. The copper complex salts of pyrrole compounds are more stable towards acid than those of zinc. The stronger HCl however splits the compound and sets free the bile pigment which has been changed partly into a biliviolin. Whether this more rapid change of the copper salt is due to an oxidation by bivalent copper or occurs as an effect of the stronger acid on the copper complex is not clear.

If ammonia were added the olive copper compound gave a blue solution showing the typical bands of complex salts of the biliviolin type at 632 and 578 $m\mu$.

Biliverdin, uteroverdin and oocyan dimethyl esters.

In the paper of Lemberg and Barcroft [1932] Bernal reported that oocyan and uteroverdin esters differed in the optical properties of the crystals. I found later a similar difference between the crystals of uteroverdin ester and biliverdin ester obtained from bilirubin with ferric chloride.

Different preparations of natural and synthetic biliverdin esters have been examined. All crystallise in one of the two forms which may be called A and B or they may contain a mixture of both. Form A is that observed by Bernal for oocyan ester, form B that found by him for uteroverdin ester. In Bernal's report the orientation of the pleochroism of both forms was given inversely to that observed now, which was probably due to a mistake.

Pure A was obtained if pure bilirubin was dehydrogenated whether with ferric chloride or with atmospheric oxygen in alkaline solution. Even impure samples of the ester crystallised then in the A-form only. The purest was one

obtained by esterification of pure crystalline biliverdin; its M.P. was 223° (corr.). But samples melting as low as 195° crystallised as A.

Very small amounts of B were obtained from pure bilirubin twice. Once bilirubin was extracted with boiling glacial acetic acid. A very small amount went into solution and was simultaneously dehydrogenated to biliverdin which gave B on esterification. Again a very small sample of B was got from the ethereal mother-liquor of biliverdin, after the chief part of the biliverdin had separated.

B was predominant in uteroverdin ester, some samples of which were almost free from A. The ester of oocyan contained more A and less B. Mixtures of A and B resulted also from biliverdin extracted from ox gallstones by extraction with boiling glacial acetic acid or by dehydrogenation of bilirubin, which had been prepared from the mother-liquors in chloroform by precipitation with alcohol. Here again the melting-point varied from 196° up to 209° (for uteroverdin ester) and to 215° for the product of the glacial acetic acid extract of gallstones. There was no melting-point depression on mixing the different A or B esters or A with B ester.

No change could be observed from B to A or reverse, on recrystallisation. If a mixture of A and B were fractionated by crystallisation from methanol, A crystallised first, B from the mother-liquors.

The mesobiliverdin synthesised from analytical neoxanthobilirubic acid [Fischer *et al.* 1932] is according to Siedel and Fischer [1933] a mixture of the natural (unsymmetrical) mesobiliverdin with two other symmetrical mesobiliverdins with different position in the side-chains. The crystals of the synthetic mesobiliverdin ester were compared with crystals of that obtained from bilirubin ("natural ester").

Natural ester: oblique extinction; pleochroism extraordinarily strong (NS), violet-grey (EW), blue; at 45°, red-violet.

Synthetic ester: Extinction changing from almost straight to oblique. Pleochroism different in different crystals, partly similar to the "natural" product, partly much less distinct.

Although these facts seem to favour the assumption that A and B forms are structurally different, the exact investigation of the optical properties of the crystals showed that the differences can be explained satisfactorily as due to growth and orientation of the crystals; a particular impurity may cause the substance to crystallise in the B form.

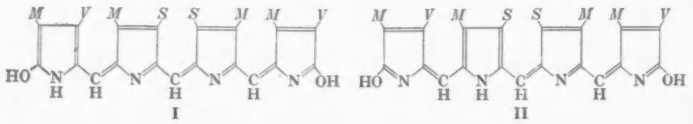
If the crystals are monoclinic, then A may be a form in which the crystals are tabular parallel to (110) or another vertical face elongated along *c*. The extinction would then be oblique. Tilting the crystal along its long axis into the direction in which the light is perpendicular to (100) should give straight extinction, turning it in the opposite direction should increase the angle of extinction up to the value it shows for its (010) plane. That is what actually happens if the A-crystals are tilted on a hemisphere.

The B-crystal may be one showing its (100) or (001) plane. The different pleochroism can be explained by the fact that it is elongated along *b*. Thus on turning the extinction remains straight.

Further evidence against the assumption of a genuine difference between A and B arises from the investigation of the hydrochloride crystals. No difference could be found between the chlorides of pure A-ester and of uteroverdin ester. Both show the following properties: flat needle-shaped green prisms; complete extinction almost straight (deviation less than 2°). Pleochroism: (NS) blue-green (dark in sodium light), (EW) green (lighter in sodium light), slow wave parallel to long axis. The partial birefringence can be roughly estimated to be 0·02.

Discussion.

Two formulae have been discussed for biliverdin [Fischer and Adler, 1931; Lemberg, 1932; Siedel and Fischer, 1933; Lemberg and Bader, 1933] which differ in the position of the double bonds in the rings I and II:



I II

I had preferred formula I in 1932, as biliverdin was then thought to be incapable of forming complex salts, and this formula accounted better for their non-existence. But as complex salts have now been proved to exist, there is no longer any reason for preferring it. Furthermore a substance of formula I should form dichlorides (as the porphyrins do) on its two middle basic nitrogen atoms, whereas in formula II a monochloride, which has been found, is understandable; the two nitrogen atoms of the rings I and IV are less basic being lactim-nitrogen (—N=C(OH)—).

Mesobiliverdin was considered by Siedel and Fischer [1933] to be the blue stage of the Gmelin test. It is shown in this paper that this pigment is the chief product in the green phase of the Gmelin test, whereas it is hardly present when the blue phase is reached. The Gmelin test was carried out at high dilution which favours the successive formation of the single reaction phases. Nevertheless mixtures were obtained. They were separated by use of the Willstätter HCl-number method, which is as applicable to certain types of bile pigments as to porphyrins or chlorophyll derivatives.

Mesobiliverdin has been obtained in the form of the ferric chloride double salt of its ester chloride by oxidation of mesobilirubinogen with ferric chloride. The bearing of this observation on the relation between the green and violet bile pigments (mesobiliviolin and the phycobilins of red algae) and on the urobilin problem will be discussed in a forthcoming paper. A short report has been already given [Lemberg, 1934].

Summary.

The preparation is described of dehydromesobilirubin dimethyl ester from crude "mesobiliverdin" and of dehydrobilirubin ester from "biliverdin." It is proposed that the names biliverdin and mesobiliverdin should be used for the green dehydrobilirubins.

Mesobiliverdin forms the first (green) stage of the Gmelin reaction, from which it could be isolated in a yield of 36 % of the mesobilirubin which entered the reaction.

Mesobiliverdin is obtained from mesobilirubinogen by oxidation with ferric chloride in the form of the ferric chloride double salt of its dimethyl ester chloride ($C_{35}H_{42}O_6N_4 . HCl . FeCl_3$).

The method of preparation of biliverdin is improved and its formula $C_{33}H_{34}O_6N_4$ (with two hydrogen atoms less than bilirubin) is confirmed.

Biliverdin dimethyl ester can crystallise in two different forms. The first form is obtained from pure bilirubin. Natural biliverdins (uteroverdin and oocyan) give a second form mixed with more or less of the first; so does biliverdin from the mother-liquors of bilirubin. The two types can be explained by differences of crystal growth and the crystallographic investigation gives no evidence

of the existence of two structurally different substances. Biliverdin, uteroverdin and oocyan may be considered as identical.

Crystalline monochlorides are obtained from biliverdin, mesobiliverdin and their esters. The biliverdins form complex salts with Zn and Cu, but the Zn salt lacks the typical fluorescence of pyrrole pigment zinc complexes. These observations allow of a decision between the two formulae which have been proposed for biliverdin.

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CXXXVIII. A COLORIMETRIC METHOD FOR THE DETERMINATION OF *N*-ACETYLGLUCOSAMINE AND *N*-ACETYLCHONDROSAMINE.

By WALTER THOMAS JAMES MORGAN and LESLIE ALDERMAN ELSON[1].

From the Serum Department of the Lister Institute, Elstree, Herts.

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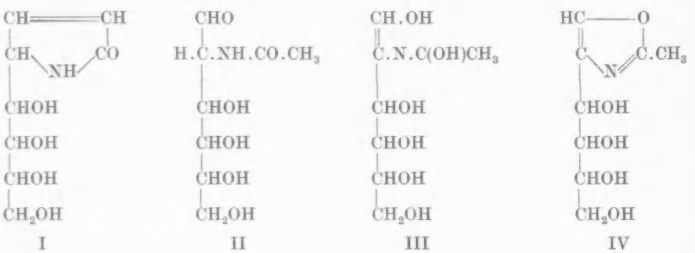
The reaction of acetylated glucosamine with *p*-dimethylaminobenzaldehyde was first observed by Müller [1901] who showed that after warming a solution of penta-acetylglucosamine with dilute potassium hydroxide an intense reddish-purple colour was produced by the addition of a solution of *p*-dimethylaminobenzaldehyde acidified with hydrochloric acid [Ehrlich's reagent; 1901].

A colorimetric method for the quantitative estimation of *N*-acetylglucosamine, based on the above reaction, has recently been described by Zuckerkandl and Messiner-Klebermass [1931]. These workers suggested that when *N*-acetylglucosamine (II) is warmed with dilute alkali the aldehyde group and the acetyl group react together with the subsequent formation of a pyrrole derivative (I) which then condenses with *p*-dimethylaminobenzaldehyde to yield the characteristic red coloration which pyrroles are known to give with Ehrlich's reagent. We have obtained evidence [Elson and Morgan, 1933, 1, 2, and unpublished results], however, that *N*-acetylglucosamine reacts in the enolic form (III) and passes over with loss of water into the oxazole derivative, 2-methyl-4-$\alpha\beta\gamma\delta$-tetrahydroxy-*n*-butyloxazole (IV), and we consider that it is the condensation of this substance with *p*-dimethylaminobenzaldehyde in acid solution which gives rise to the intense reddish purple coloration. The action of dilute alkali on *N*-acetylglucosamine will be considered in more detail in a later communication.



According to the procedure of Zuckerkandl and Messiner-Klebermass *N*-acetylglucosamine (1–4 mg.), after dissolution in 5 ml. of 60 % aqueous alcohol, is heated for 6–8 seconds at boiling-point with two drops of 30 % potassium hydroxide solution and rapidly cooled. The *p*-dimethylaminobenzaldehyde reagent (1 ml.) is then added and after 10–15 minutes the reddish-

[1] Jenner Research Scholar.

purple colour which develops is matched colorimetrically against the colour produced by a standard solution of *N*-acetylglucosamine which has been treated in the same manner.

In our experience this method cannot be used where accurate and reproducible results are required. It is not proposed to discuss here the numerous variations in technique which have been tried in order to render the method suitable for our requirements, but only to state that investigations were carried out to determine the optimum conditions under which *N*-acetylglucosamine could be converted into the oxazole derivative (IV) and to ascertain the factors which are necessary for the production of the maximum colour intensity per mg. of the oxazole derivative when this compound is condensed with *p*-dimethylaminobenzaldehyde.

Early in our work it was found that unless the operation of boiling the acetylglucosamine in alkaline alcoholic solution were very carefully controlled a considerable proportion remained unconverted into the oxazole derivative or was destroyed by the action of the alkali. In view of the marked influence of potassium hydroxide upon the quantitative conversion of the *N*-acetylhexosamine into the corresponding oxazole under these conditions we consider that—"two drops of 30 % potassium hydroxide"—and—"heating for 6–8 seconds at boiling-point"—as recommended by Zuckerkandl and Messiner-Klebermass, do not define the concentration of alkali present in the test solution, or the time of heating, with sufficient accuracy for a quantitative procedure.

A greater colour intensity per mg. of acetylglucosamine results when the colour reaction is carried out in 90 % acetic acid solution and by the use of this solvent it has been possible to estimate quantities of acetylglucosamine as small as 0·08 mg. A perceptible colour is obtained with 0·01 mg. acetylglucosamine in a volume of 10 ml.; the test is therefore sensitive at a dilution of 1 in a million of acetylglucosamine.

Experimental.

Reagents.

(1) p-*Dimethylaminobenzaldehyde reagent.* *p*-Dimethylaminobenzaldehyde (A. R.) (2 g.) after two crystallisations from dilute acetic acid is dissolved in 100 ml. of glacial acetic acid containing 5·0 ml. of hydrochloric acid (A. R.). The final solution should possess a pale yellow colour. The addition of 1 ml. of water to 9 ml. of the reagent should not produce an increase in the intensity of the yellow colour. Some preparations of *p*-dimethylaminobenzaldehyde (A. R.) have been found to give yellow-coloured acetic-hydrochloric acid solutions which produce bright yellow solutions on the addition of water; these must be avoided. The reagent keeps indefinitely.

(2) *N-Acetylglucosamine standard.* *N*-Acetylglucosamine (1 g.) is dissolved in about 10 ml. of water saturated with chloroform and made up to a volume of 100 ml. From this standard solution dilutions which will contain a required amount of substance per ml. can readily be made. The standard solution should be kept at 0°.

(3) *0·5 N sodium carbonate solution.*

Procedure.

A solution of *N*-acetylglucosamine, containing 0·1–1·0 mg. per ml., is measured into test-tubes of approximately equal thickness graduated at 10 ml.; if the volume of solution taken is less than 1·0 ml. it should be made up to 1·0 ml. by the addition of water. The sodium carbonate (0·1 ml.) is added and

the tubes are heated in a boiling water-bath for 5 minutes and then cooled. Standard solutions of *N*-acetylglucosamine are treated in the same manner. Glacial acetic acid is run into the tubes from a burette or separating funnel until it is within about 2 ml. of the 10 ml. mark. The *p*-dimethylaminobenzaldehyde reagent (1 ml.) is then added and the solution is made up to 10 ml. with acetic acid, thoroughly mixed and allowed to stand. The colour develops quickly and reaches its maximum intensity in about 45 minutes. When the maximum colour reached is compared with a stable artificial colour standard it shows no appreciable fading over a period of at least 1 hour.

As with most colorimetric methods, this method gives good results only when the colour intensities of the standard and the unknown solutions are approximately the same. Table I shows the results obtained by using the above

Table I.

N-Acetylglucosamine				*N*-Acetylglucosamine			
Standard mg.	Present mg.	Found mg.	% error	Standard mg.	Present mg.	Found mg.	% error
0·90	1·00	1·00	0·0	0·400	0·350	0·355	+1·4
,,	0·95	0·96	+1·0	,,	,,	0·350	0·0
,,	,,	0·97	+2·1	0·350	0·400	0·405	+1·2
,,	0·85	0·84	−1·2	,,	0·300	0·310	+3·3
,,	,,	0·86	+1·2	0·325	0·350	0·345	−1·4
,,	,,	,,	+1·2	,,	0·300	0·305	+1·7
,,	0·80	0·81	+1·3	0·300	0·275	0·275	0·0
0·80	0·90	0·89	−1·1	,,	0·250	0·255	+2·0
,,	0·70	0·70	0·0	0·225	,,	,,	+2·0
0·70	0·80	0·78	−2·5	0·175	0·200	0·205	+2·5
,,	,,	0·79	−1·2	0·150	0·175	0·174	−0·6
,,	0·65	0·64	−1·5	,,	,,	0·177	+0·6
,,	0·60	0·62	+3·3	0·090	0·100	0·102	+2·0
,,	,,	0·61	+1·6	,,	,,	0·099	−1·0
0·55	,,	0·60	0·0	,,	,,	0·101	+1·0
,,	,,	0·585	−2·5	,,	0·080	0·078	−2·5
,,	0·50	0·510	+2·0	,,	,,	,,	−2·5
,,	,,	0·500	0·0	,,	,,	0·079	−1·2
0·450	,,	0·510	+2·0				
,,	,,	0·495	−1·0				
,,	0·40	0·395	−1·2				

procedure when the solutions to be estimated differ in their *N*-acetylglucosamine content by not more than 20 % from that of the standard solution. The method is equally applicable to the estimation of *N*-acetylchondrosamine and it has been

Table II.

N-Acetylchondrosamine			
Standard mg.	Present mg.	Found mg.	% error
0·90	1·00	1·02	+2·0
,,	,,	1·00	0·0
,,	0·85	0·84	−1·2
,,	,,	0·86	+1·2
0·60	0·65	0·65	0·0
,,	,,	0·64	−1·5
,,	0·55	0·56	+1·8
0·550	0·500	0·505	+1·0
0·400	0·450	0·445	−1·1
,,	0·350	0·365	+4·3
,,	,,	0·360	+2·9
0·090	0·100	0·101	+1·0
,,	,,	0·102	+2·0

found that equal amounts of *N*-acetylglucosamine and *N*-acetylchondrosamine when compared under similar conditions give rise to colours identical in tint and intensity. A few results of estimations carried out with *N*-acetylchondrosamine solutions are given in Table II.

A comparison between the results obtained with the original method of Zuckerkandl and Messiner-Klebermass and those obtained using the new technique is shown in Table III. In each series of determinations the results of

Table III.

Experiment no.	Zuckerkandl and Messiner-Klebermass's method 2·00 mg. acetylglucosamine		New technique 0·500 mg. acetylglucosamine	
	Found mg.	% error	Found mg.	% error
1	2·12	+6·0	0·502	+0·4
2	2·16	+8·0	0·498	−0·4
3	1·95	−2·5	0·500	0·0
4	1·95	−2·5	0·498	−0·4
5	2·10	+5·0	0·492	−1·6
6	1·98	−1·0	0·502	+0·4
7	2·18	+9·0	0·500	0·0
8	1·94	−3·0	0·500	0·0
9	1·91	−4·5	0·490	−2·0
10	2·03	+1·5	0·502	+0·4
11	2·00	0·0	0·505	+1·0
12	2·08	+4·0	0·500	0·0

twelve consecutive estimations are given. It will be seen that with the old method the variation between the individual results is of the same order as that observed by Zuckerkandl and Messiner-Klebermass and may exceed 5 %. The results obtained with the modified technique show a variation of only 1–2 % from the true value.

Table IV. *The influence of foreign substances upon the accuracy of the estimation.*

Substance	Added mg.	*N*-Acetylglucosamine hydrochloride: Present mg.	Found mg.	% error
Glucose	1·0	0·500	0·500	0·0
,,	,,	,,	0·505	+1·0
,,	2·5	0·250	0·245	−2·5
Arabinose	1·00	0·500	0·500	0·0
,,	,,	,,	0·500	0·0
Fructose	1·00	0·500	0·500	0·0
,,	,,	,,	0·505	+1·0
,,	2·50	0·250	0·240	−4·0
Alanine	1·00	0·500	0·510	+2·0
,,	,,	,,	0·500	0·0
Histidine	,,	,,	0·495	−1·0
,,	,,	,,	0·510	+2·0
Glucosamine hydrochloride	,,	,,	0·505	+1·0
,,	,,	,,	0·500	0·0
1-Aminoglucose	,,	,,	0·500	0·0
,,	,,	,,	0·510	+2·0
N-Acetyl-1-aminoglucose	,,	,,	0·500	0·0
,,	,,	,,	0·505	+1·0

The influence of foreign substances.

Various sugars and amino-acids have been added to known amounts of *N*-acetylglucosamine in order to ascertain whether the presence of these substances has any influence upon the accuracy of the estimation. A few results of these tests are given in Table IV from which it will be seen that none of the substances added interfered with the determination.

Certain pyrrole and indole derivatives develop coloured solutions when treated with the *p*-dimethylaminobenzaldehyde reagent, and their presence in the solutions to be estimated will therefore influence the determination. Tryptophan does not appear to react with *p*-dimethylaminobenzaldehyde and does not yield a coloured solution under the conditions described, but the presence of moderate amounts (0·1–1·0 mg.) of this amino-acid in the *N*-acetylglucosamine solutions has been found to cause some delay in the rate of development of the reddish purple colour with resulting underestimation of the true result.

The influence of the time of heating and of the concentration of alkali on the subsequent colour development.

Estimations were made on solutions containing 0·5 mg. of *N*-acetylglucosamine per ml. The concentration of the alkali varied from 0·02 *N* to 2 *N* and three or four different periods of heating were tested with each alkali concentration. It is not necessary to give here the results obtained with all of these modifications but only to state that with concentrations of alkali higher than 0·5 *N* the solution, after heating at 100° for more than 2 minutes, developed a final colour intensity which indicated that a considerable destruction of either the *N*-acetylglucosamine or the corresponding oxazole derivative had taken place. The results obtained using 0·02 *N* and 0·1 *N* alkali are shown in Table V. It will be seen that the greatest colour intensity is attained when the *N*-acetylglucosamine

Table V. *Influence of the time of heating with various concentrations of alkali on the subsequent colour development.*

Alkali used	Concentration of alkali *N*	Time of heating min.	Colorimeter reading at maximum colour developed mm.
Na_2CO_3	0·02	5	9·0
,,	,,	10	**8·8**
,,	,,	15	8·9
,,	0·10	2	11·5
,,	,,	3	9·3
,,	,,	4	9·1
,,	,,	5	**9·0**
,,	,,	6	9·3
,,	,,	10	13·2
,,	,,	15	18·9
KOH	0·02	2	12·5
,,	,,	3	**10·1**
,,	,,	4	10·6
,,	,,	5	11·4
,,	0·10	2	10·2
,,	,,	3	13·2
,,	,,	4	20·6
Na_2HPO_4	0·10	5	21·6
,,	,,	10	17·2
,,	,,	30	14·8
,,	,,	40	**13·2**

is heated for 10 minutes at 100° in 0·02 *N* Na_2CO_3. If 0·02 *N* KOH is used in place of the 0·02 *N* Na_2CO_3 the maximum colour intensity which is reached for the same amount of *N*-acetylglucosamine is somewhat less. This is due to the action of the potassium hydroxide on the oxazole derivative, and the exact extent to which decomposition proceeds can readily be ascertained if a standard solution of the pure oxazole (IV) is treated with alkali under similar conditions.

The production of the oxazole derivative by heating with 0·02 *N* Na_2CO_3 appears to be almost quantitative. To show this a solution of maximum colour intensity which has been derived from a known amount of *N*-acetylglucosamine is compared colorimetrically with the colour developed by a solution of the pure oxazole which has been similarly treated with the *p*-dimethylaminobenzaldehyde reagent. Theoretically 0·5 mg. of *N*-acetylglucosamine should yield 0·459 mg. of the oxazole derivative. After treatment with 0·02 *N* Na_2CO_3 as described above, 0·5 mg. of *N*-acetylglucosamine gave a solution the colour intensity of which was equal to the colour given by 0·440 mg. of the oxazole; the conversion was therefore 96 % complete.

The production of a closed ring structure by the action of very low concentrations of alkali suggested that the reaction would probably take place at an alkalinity which could be considered to be within the range necessary for normal biological processes. The results obtained when *N*-acetylglucosamine was heated with 0·1 *N* Na_2HPO_4 (p_H 8·4) are shown in Table V. It will be seen that approximately 65 % of the *N*-acetylglucosamine has been converted into the oxazole derivative after 40 minutes' heating at 100°. A sterile solution of *N*-acetylglucosamine which has been buffered at p_H 8·4 with sodium phosphate and kept at 37° showed after 48 hours that about 10 % had been converted into the oxazole.

Although the maximum colour intensity reached per mg. of *N*-acetylglucosamine is slightly less when 0·1 *N* Na_2CO_3 is used in place of 0·02 *N*, we consider that for practical purposes a higher concentration of alkali than 0·02 *N* is more reliable since it is then possible to neglect slight traces of acid when these are present in the solution to be estimated.

The influence of the concentration of the hydrochloric acid upon the colour intensity.

A series of eight test-tubes containing *N*-acetylglucosamine (0·5 mg.) dissolved in 1·0 ml. of water was heated with 0·1 ml. of 0·5 *N* Na_2CO_3 for 5 minutes in a boiling water-bath and cooled. The tubes were then treated one at a time in the following manner. About 7·0 ml. of glacial acetic acid were added to each together with 0·1 ml. 0·5 *N* HCl and 1·0 ml. of *p*-dimethylaminobenzaldehyde (2 %) dissolved in glacial acetic acid which contained in the different experiments concentrations of hydrochloric acid[1] varying from 1 to 40 %. The tubes, after filling to the 10 ml. mark with acetic acid, were thoroughly shaken and the intensity of the colour produced was followed in each case by means of colorimetric observations at prearranged intervals against a stable artificial colour standard for a period of 90 minutes. The results are shown graphically in Fig. 1. It is seen that a reduction in the concentration of hydrochloric acid from 40 to 2·5 % in the *p*-dimethylaminobenzaldehyde reagent causes a steady increase in the intensity of the maximum colour produced; the rate of colour development is however slower with the lower acid concentrations and the colour formed is considerably more stable. When the reagent contains 20 or 30 % HCl, the

[1] Expressed throughout as % of concentrated HCl A. R. present.

intensity of the reddish purple colour reaches its maximum in less than 10 minutes and then rapidly fades. A concentration of hydrochloric acid of 5 % in the reagent causes the colour intensity to develop for about 45 minutes and then remain practically constant for a further 60 minutes. The colour development when 2·5 % of hydrochloric acid is employed is very slow, and the intensity of the colour developed does not reach its maximum until after about 90 minutes have elapsed; 1·0 % of acid in the reagent produces only a trace of colour after 2 hours. It should be emphasised however that the rate of colour development increases rapidly with increasing temperature and that the curves shown were obtained within the temperature range 13–16°.

From these observations it appears that the colour is very sensitive to an excess of hydrochloric acid and that, with concentrations of acid exceeding 0·5 % in the final acetic acid solution, fading of the colour goes on concurrently with colour production.

A similar series of curves is obtained when standard solutions of the pure oxazole derivative (IV) are treated in the same manner with the *p*-dimethylaminobenzaldehyde reagent containing different concentrations of hydrochloric acid.

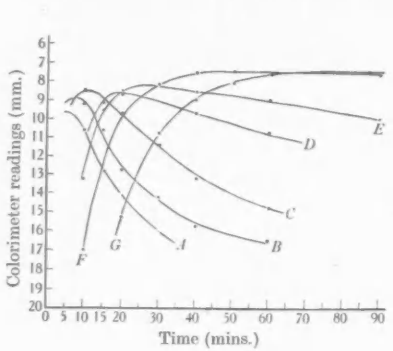


Fig. 1.

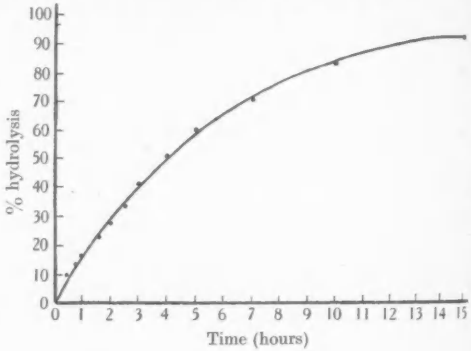


Fig. 2.

Fig. 1. The influence of the concentration of hydrochloric acid on the rate and intensity of colour development. Curve *A* 40 %, *B* 30 %, *C* 20 %, *D* 15 %, *E* 10 %, *F* 5 %, *G* 2·5 % of hydrochloric acid in the *p*-dimethylaminobenzaldehyde reagent.

Fig. 2. Curve showing rate of hydrolysis of *N*-acetylglucosamine with 0·1 *N* sulphuric acid.

Application.

The method has been used to follow the rate of hydrolysis of the acetyl group in *N*-acetylglucosamine, and the results of a typical hydrolysis experiment are shown in Fig. 2. The final figure shows that 94 % of the acetyl groups had been eliminated in 15 hours, and this figure was confirmed by estimation of the glucosamine formed using the colorimetric method described by Elson and Morgan [1933, 1]. Similarly the rate of esterification of *N*-acetylglucosamine to yield the non-reducing 1-methyl-*N*-acetylglucosamine and the hydrolysis of this compound back to *N*-acetylglucosamine can be readily followed by the colorimetric method described. The procedure has also been used to determine the rate at which the *N*-acetylhexosamine units, which are contained in the specific polysaccharide of *B. dysenteriae* (Shiga), are liberated by acid or enzymic hydrolysis [Morgan, 1931; 1932; Morgan and Thaysen, 1933].

SUMMARY.

A colorimetric method for the determination of *N*-acetylglucosamine, which was elaborated by Zuckerkandl and Messiner-Klebermass and which is based on a reaction originally described by Müller, has been studied. The procedure recommended by them has been found to give unsatisfactory results. By changing the technique it is possible to obtain reproducible results and at the same time to reduce considerably the amount of substance necessary for the determination. *N*-Acetylchondrosamine can be estimated by the modified method.

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CXXXIX. ON THE NATURE OF THE PRECURSOR OF THE VITAMIN C IN THE VEGETABLE KINGDOM.

I. VITAMIN C IN THE GROWING PEA SEEDLING.

By SURENDRA NATH RAY.

From the Biochemical and Nutritional Laboratories, University of Cambridge.

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In an earlier paper [Harris and Ray, 1933], it was shown that ascorbic acid appears in pea seeds as soon as germination begins. Since then the rate of production of ascorbic acid in the seedlings of such pea seeds has been examined, and the result is embodied in Table I. For the estimation of the acid, the

Table I.

Period of germination	Wt. of 20 seedlings g.	Amount of ascorbic acid mg./g. seedlings
12 hours (seeds kept soaked in water)	0·18	0·00
48 hours (germinated)	0·29	0·23
60 ,, ,,	0·60	0·43
90 ,, ,,	1·70	0·50
115 ,, ,,	2·60	0·50
140 ,, ,,	2·50	0·48
170 ,, ,,	4·2	0·49
12 days ,,	4·8	0·49

micro-chemical method of Birch *et al.* [1933] was used. Yellow-pea seeds were kept in sand slightly moistened with water, and after various periods of germination were taken out and washed with water to remove the adhering sand, and the cotyledons were excised away. The separated portions (here termed the seedlings) were dried between two filter-papers and ground up with a little sand and sufficient 20 % trichloroacetic acid to give a final concentration of the latter of about 5 %. The extracts were made up to a suitable volume and filtered, and the filtrates were titrated against a standard solution of 2 : 6-dichlorophenolindophenol. As a rule, 20 seedlings were used for each estimation.

As will be noticed, the amount of ascorbic acid present per g. of the wet seedling tissue increases within a few hours from 0 to a constant value which is not affected by any further growth. This is represented graphically in Fig. 1.

It may, however, be remarked that though the young seedlings are quite rich in vitamin C, the whole of the vitamin content is not confined to them. Judged from separate titration results on the seedlings and the cotyledons, the latter have been found to account for more than 5/6 of the total reducing power. It is doubtful, however, whether the whole of the reducing power of the cotyledons is due to ascorbic acid. It is known that a few substances like cysteine reduce the dye under the conditions of the experiment. As extracts made from the cotyledons show a marked nitroprusside reaction, and as Van Eekelen *et al.* [1934] have

shown that part of the reducing material in the cotyledons is precipitated by mercuric acetate, it seems probable that some free cysteine may be present in them. Johnson [1933] has also found that extracts from germinating peas show much less antiscorbutic value than was to be expected from the titration results. On the other hand extracts from seedlings alone show no nitroprusside reaction,

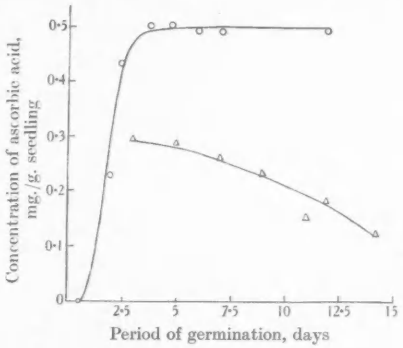

Fig. 1. Curve showing rate of production of ascorbic acid during various periods of germination or cultivation.

o, seedlings grown naturally with the cotyledons.
Δ, seedlings grown artificially on a medium containing fructose.

and they reduce the indicator solutions as rapidly as those of pure ascorbic acid solutions. These findings confirm those of Johnson, who also believes that the reducing power of seedlings is due to ascorbic acid alone.

Nature of the precursor of vitamin C in plants.

Though the synthesis of vitamin C during germination has been known for a long time, no attempt seems to have been made to ascertain the nature of the substance or substances from which it is formed. In their classical work Brown and Morris [1890] had shown that embryos of barley, excised out of the endosperm and cultivated upon nutrient solution, grew steadily at the expense of the carbohydrates or other organic nutrient substances present in the solution. It was thought that by utilising this method, *i.e.* by growing the embryo seedlings of peas on nutrient solutions containing various organic compounds and examining in the different samples whether ascorbic acid is formed or not, some information about the nature of the precursor might be obtained. Experiments in these directions have been successful and some very interesting results have been obtained. These will be discussed below.

Experimental.

The mode of experiment usually adopted was to place the excised embryo seedlings on a suitable semi-solid medium. It is essential that the embryo seedlings should rest on the medium and not be immersed in it, as in the absence of air growth and production of ascorbic acid do not take place.

It was found that a 10 % gelatin mixture constitutes an ideal medium for the cultivation of the seedlings. Gelatin itself is not assimilable by the young

plant and the 10 % solution forms a suitable semi-solid gel, whose viscosity is sufficient to prevent the embryo seedlings from sinking in it, but not great enough to stop the radicles from piercing it and absorbing the proper nutrient substances. In order to provide sufficient nitrogen and other elements necessary for a growing plant, the gelatin was dissolved in the proper quantities of the following solution (Knopp's solution):

Calcium nitrate	0·8 g.
Potassium nitrate	0·2 g.
Potassium dihydrogen phosphate ...	0·2 g.
$MgSO_4, 7H_2O$	0·2 g.
Ferric phosphate	Trace
Water	1000 ml.

The various organic substances to be tested were dissolved in this solution in a concentration of 5 %.

At first, great difficulty was experienced in keeping the medium free from bacterial contamination. If so infected, the seedlings failed to grow and little or no ascorbic acid could be detected in them. The following procedure was, however, found to give satisfactory results, and in most cases no appearance of bacterial life or signs of fermentation could be seen for the experimental period of 7 days.

1 g. of purified gelatin is dissolved in 10 ml. of the Knopp's solution containing 0·5 g. of the experimental compound. While still hot and liquid, the gelatin mixture is transferred to a 25 ml. wide-mouthed flask, fitted with a cotton-wool stopper. The flask with the mixture is then sterilised by steaming for half an hour on 3 successive days. It is then left aside for the gelatin to set. In certain cases where it was feared that the drastic steaming process might hydrolyse or decompose the experimental organic substance, the latter was not added at first, but after the gelatin mixture (8 ml.) had been sterilised the compound was dissolved in a small amount (2 ml.) of sterilised Knopp's solution and was added to the gelatin mixture before it had set. Such cases are marked in Table II with an asterisk.

Before the embryo seedlings are excised, the pea seeds are soaked in sterile distilled water for 6 hours. The seed-coats are then removed and with a little pressure the two cotyledons can be separated easily and the embryo seedling taken out by means of a pair of forceps with bent heads. The seedlings are then washed with several changes of sterilised distilled water, and finally placed on the solidified nutrient gelatin. These operations of washing and transference should be carried out as far as practicable in a dust-free room.

The seedlings after suitable periods of cultivation (usually 7 days unless otherwise mentioned) are taken out and suspended in warm water (37°) to remove any adhering gelatin. They are then dried between two filter-papers and extracted as usual with sand and trichloroacetic acid. The ascorbic acid in the extracts is estimated by titration against the standard 2 : 6-dichlorophenol-indophenol solution. The results are given in Table II.

From the data (Table II) it may be seen that while little reducing substance is produced in the embryo seedling when kept at about 20° on 10 % nutrient gelatin alone, a considerable amount appears when certain substances are introduced into the medium. Of these, hexoses seem to function best. The relative ascorbic acid-producing power of the individual hexoses seem to be more or less the same, mannose being, however, an exception, the seedlings being able to generate much more ascorbic acid from mannose than from any of the other three hexoses

Table II. *Results after 7 days' cultivation.*

Nature of the medium	Wt. of 20 seedlings	Amount of ascorbic acid, mg./g. wet seedling tissue
10 % gelatin solution alone	0·49	0·049
" " "	0·46	0·050
" " +glucose	0·87	0·194
" " "	0·40	0·250
" " "	0·80	0·225
" " +fructose	0·80	0·300
" " "	1·03	0·281
" " " *	0·93	0·264
" " +galactose	0·48	0·237
" " "	0·85	0·168
" " " *	0·55	0·201
" " +mannose	0·37	0·540
" " "	0·36	0·691
" " +sucrose	0·98	0·275
" " "	0·55	0·320
" " " *	0·60	0·310
" " +lactose	0·60	0·130
" " +maltose	1·15	0·208
" " +arabinose	0·63	0·0
" " " *	0·50	0·0
" " +xylose	0·74	0·120 (?)
" " " *	0·63	0·027
" " " *	0·60	0·054
" " +starch	0·57	0·061
" " +dextrin	0·78	0·078
" " +mannitol	0·43	0·039
" " +sorbitol	0·43	0·0
" " +inositol	0·50	0·054
" " +sodium pyruvate	0·15	0·0
" " +methylglyoxal	0·15	0·0
" " +sodium glutamate	0·20	0·0
" " +glycerol	0·92	0·079
" " +sodium lactate	0·18	0·0
" " +sodium glycerophosphate*	0·20	0·0

* Substance added to gelatin afterwards (see p. 998).

examined. Disaccharides come next in the list but here it is possible that ascorbic acid is not derived from the disaccharides themselves but from the products of their enzymic hydrolysis. Polysaccharides seem to yield a small, though definite, amount of ascorbic acid, but here again it is doubtful whether they themselves or secondary products are responsible. Pentoses yield consistently negative results; the high value obtained in one case from xylose is probably due not to xylose but to the decomposition products arising during the drastic process of long steaming. Of various other compounds examined only glycerol seems to have a little power while all the rest either gave absolutely negative values or values equal to that given by control seedlings grown on nutrient gelatin alone.

It seems very probable, therefore, that ascorbic acid may be produced by the growing embryo from the hexoses present in the seeds. On the other hand, the concentration of ascorbic acid in the seedlings germinated on hexose media is rather low (0·2–0·3 mg. as compared with 0·5 in seedlings grown with their natural cotyledons); mannose, however, is an exception, the concentration of ascorbic acid being similar to that found under natural conditions. These rather low values may mean that the real precursor is perhaps a substance different from the hexoses. If such a substance exists, however, it is quite evident that its addition is not essential for the production of ascorbic acid and that embryo seedlings are well equipped with enzyme systems to convert ordinary hexoses into this hypothetical substance. About the course of the conversion of hexoses

into ascorbic acid nothing is known. Attempts to grow the seedlings on media containing some of the well-known carbohydrate breakdown products have failed (see Table II). The presence of these substances in the nutrient gelatin seems to inhibit growth, and no trace of ascorbic acid can be detected in the seedlings. Similarly all efforts to elucidate the nature of the enzyme system responsible for this synthesis have been unsuccessful. Attempts to produce ascorbic acid from sugar solutions by means of minced or crushed embryo seedling tissue have only given negative results.

Another interesting thing to be observed from Table II is that there seems to be no direct relation between extent of growth and production of ascorbic acid. Thus the seedlings which are grown on mannose show very little growth, whereas they are exceptionally rich in ascorbic acid. On the other hand, arabinose, xylose, dextrin, all seem to favour growth, but the production of ascorbic acid in these cases is either nil or very low. Substances like fructose seem to favour both growth and production of ascorbic acid.

Antiscorbutic activity of seedlings grown on nutrient gelatin.

Though the chemical determination of ascorbic acid is fairly specific it has certain limitations and may give high values in certain cases [Birch *et al.*, 1933; Harris, 1933]. In view of this it was thought best to check the chemical estimation against direct feeding tests. The biological determination gave results in complete agreement with the indophenol titration. The seedlings used for this purpose

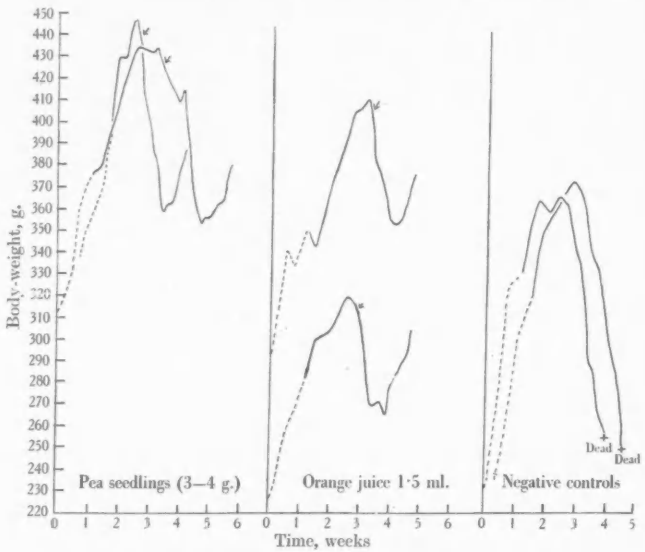

Fig. 2. Weight curves of guinea-pigs, comparing degrees of cure of scurvy with doses of pea seedling and orange-juice.

- - - - -, preliminary complete diet.
———, vitamin C-free diet.
↓, experimental dosing commenced.
+, animal died.

were grown for 7–10 days on nutrient gelatin containing fructose. As it was practicable to grow only a limited amount of these only one level of dosage could be employed. Two guinea-pigs were used for this test and the technique of assay was the curative method described in a previous paper [Harris and Ray, 1932]. A daily dose of 3–4 g. (ascorbic acid content as determined by indophenol titration being 0·75–0·9 mg.) was given to each of the animals. A control group of two guinea-pigs received 1·5 ml. of orange-juice (ascorbic acid content determined chemically about 0·9 mg.) and negative controls were kept on the basal diet alone. The individual growth curves are given in Fig. 2.

3–4 g. of the seedling were found to have nearly the same biological activity as 1·5 ml. of orange-juice. This result corresponds within the limits of experimental error with that calculated from the chemical titration. This shows that the reducing power of the seedling is due solely to the ascorbic acid.

Rate of production of ascorbic acid in seedlings grown on nutrient gelatin containing fructose.

It has already been remarked that using different substances as substrate, no correlation can be detected between growth and synthesis of ascorbic acid. It seemed of interest to examine whether any such correlation exists when the seedlings are cultivated for various periods on media containing one and the same substance. For this purpose, embryo seedlings were grown on media containing fructose (5 %) as the substrate. In order to avoid formation of secondary products during the steaming process, the fructose dissolved in a little sterilised Knopp's solution was added to the gelatin mixture after the latter had been steamed. A large number of flasks each containing 25 embryo seedlings were used and after the proper time of cultivation these were opened, 20 of the best seedlings being taken out for every day's estimation. The result is given in Table III.

Table III.

Period of cultivation days	Wt. of 20 seedlings g.	Total amount of ascorbic acid, mg. in the 20 seedlings	Ascorbic acid, mg./g. seedling
3	0·58	0·170	0·293
5	0·71	0·200	0·282
	0·63	0·180	0·285
7	0·93	0·246	0·264
9	1·35	0·318	0·235
11	1·10	0·165 (?)	0·150 (?)
12	1·20	0·216	0·180
14	1·40	0·177	0·127

It will be noticed that as a rule the greater the growth, the less was the concentration of the ascorbic acid formed, but no simple relation between the two appeared to exist; on the other hand, the concentrations when plotted against the periods of cultivation were found to form a continuous curve (see Fig. 1). The shape of the curve shows that ascorbic acid was produced in great quantities during the first 3 or 4 days of cultivation; afterwards however the rate of formation fell and became zero after about 10 days. After that period some of the ascorbic acid seemed to disappear. On the other hand, the rate of growth continued unchanged and as a consequence the concentration steadily fell. This behaviour is entirely different from that seen in seedlings grown with their cotyledons, the concentration of ascorbic acid rises in these conditions to a constant value.

Correlation between concentration of substrate and production of ascorbic acid.

A further lack of correlation between growth and the synthesis of ascorbic acid was observed when the production of ascorbic acid in seedlings grown for 7 days on media containing varied amounts of fructose was measured (Table IV).

Table IV.

Percentage concentration of sugar solution	Wt. of 20 seedlings	Total amount of ascorbic acid, mg. in the 20 seedlings	Ascorbic acid, mg./g. seedling
1·0	1·2	0·100	0·083
2·5	1·4	0·217	0·155
5·0	0·93	0·246	0·264
7·5	0·80	0·210	0·263
10·0	0·60	0·156	0·260

It is at once apparent that the rate of growth and the rate of production of ascorbic acid follow two different lines, the optimum concentration of the substrate for growth being about 2·5 %, while for the production of ascorbic acid it is much higher, about 5 %. Further the two rates plotted against the concentration form two different types of curves emphasising thereby that the two phenomena are quite different and possibly independent of each other (see Fig. 3).

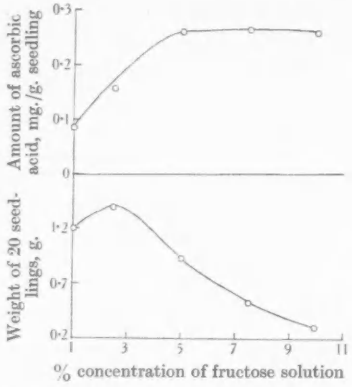

Fig. 3. Relation of rate of production of ascorbic acid by pea seedlings (upper curve) and rate of growth (lower curve) to the concentration of the substrate in the medium on which the seedlings are grown.

Cultivation of seedlings on media containing a mixture of substances.

From Table II it was seen that while some substances favour growth, others seem conducive to the production of ascorbic acid. Attempts were made to combine the two properties, *i.e.* to have good growth together with large production of ascorbic acid. For this embryo seedlings were grown for 7 days on media containing a mixture of two substances—the one favouring growth and the other production of ascorbic acid. As however the results in Table V show, these experiments have not been successful. The volume of medium was 10 ml.

Table V.

Nature of medium	Wt. of 20 seedlings, g.	Concentration of ascorbic acid, mg./g. seedling
0·1 g. fructose + 0·5 g. mannose	0·27	0·596
0·5 g. arabinose + 0·5 g. mannose	0·15	0·553

The presence of mannose seems to inhibit growth altogether.

Summary.

1. The rate of production of ascorbic acid in pea seedlings germinated naturally has been estimated. The amount formed was found to rise for 2 days and then reach a constant concentration of 0·5 mg. of the acid per g. of the wet weight of the seedlings.

2. Embryo seedlings from peas were excised from the cotyledons and grown on nutrient gelatin. Seedlings cultivated on such a medium, containing hexoses, were found to have synthesised a large amount of ascorbic acid. Of the four hexoses examined, mannose gave the highest figure, 0·5 mg. of ascorbic acid per g. of seedling. Disaccharides also gave moderately high figures but all other substances examined either gave very low or absolutely negative values. It is suggested that hexoses may serve as precursors of vitamin C in germinating seeds.

3. The rate of production of ascorbic acid in the cultivated excised seedlings bore no direct relation to the rate of growth, *e.g.* seedlings grown on mannose showed very little growth but had a high concentration of ascorbic acid while arabinose and xylose seemed to favour growth but production of ascorbic acid was nil.

4. The concentration of ascorbic acid present per g. in the excised seedlings fell steadily during prolonged periods of cultivation.

5. The concentration of ascorbic acid formed was approximately proportional to the concentration of the substrate up to a certain optimum value, after which it remained constant.

6. Unsuccessful attempts were made to produce good growth combined with large production of ascorbic acid by cultivating excised seedlings on a medium containing a mixture of two substances—one of them found to favour growth and the other production of ascorbic acid.

I am very grateful to Dr L. J. Harris for his constant advice and valuable criticism. For his kind interest in this work, I wish to thank Sir F. G. Hopkins.

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CXL. CHANGES IN THE CALCIUM AND MAGNESIUM OF THE SERUM AND IN THE INORGANIC PHOSPHORUS OF THE BLOOD OF COWS AT CALVING AND OF THE CALF DURING EARLY LIFE.

By WILLIAM MILLER ALLCROFT and WILLIAM GODDEN.

From the Rowett Research Institute, Aberdeen.

(*Received May 2nd, 1934.*)

In view of the recent work of Sjollema and his co-workers on "grass tetany," a good review of which is given by Sjollema himself [1932], and of the increasing incidence of "lactation tetany" or "Hereford disease" as referred to by Dryerre [1932], it was decided, in conjunction with Dr H. H. Green of the Ministry of Agriculture Veterinary Research Laboratory, Weybridge, to include the study of serum-Mg in normal cows throughout the reproductive cycle. We, therefore, repeated our studies [Godden and Allcroft, 1932] of the changes in the blood of the cow at calving, including determinations of serum-Mg in this series and omitting those of N.P.N., total chloride and sugar which had shown no marked alterations before.

The experimental procedure and the methods for serum-Ca and inorganic P were the same as used before except that, on this occasion, the calves were

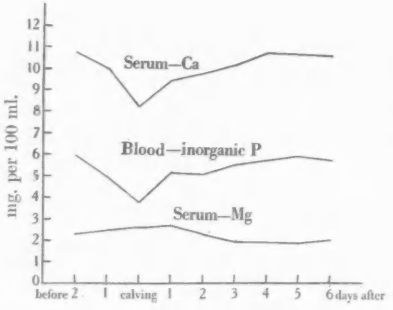

Fig. 1.

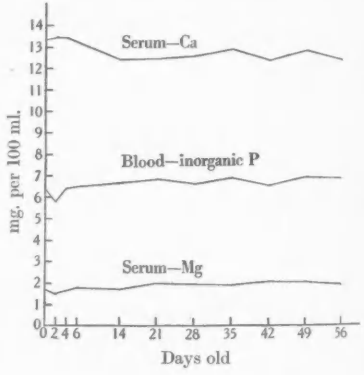

Fig. 2.

Fig. 1. Changes in the average composition of cow's blood just before, at and after calving (data for Cow 15 omitted).

Fig. 2. Changes in the average composition of the blood of 8 calves from birth to 8 weeks.

Table I. *Composition of the blood of cows, just before, at and for 6 days after calving.*

All values in mg. per 100 ml.

Days before or after calving	Serum-Ca	Serum-Mg	Blood-inorg. P	Serum-Ca	Serum Mg.	Blood-inorg. P	Serum-Ca	Serum-Mg	Blood-inorg. P	Serum-Ca	Serum-Mg	Blood-inorg. P
	Cow 4			Cow 5			Cow 7			Cow 8		
2 before	10·05	2·00	5·87	10·75	1·95	6·13	11·35	1·92	6·89	—	—	—
1 ,,	9·95	2·35	5·92	9·60	2·25	5·19	9·45	2·00	3·75	10·15	1·95	5·87
Calving	10·00	1·99	4·78	7·45	2·85	4·47	8·50	2·17	3·05	7·15	2·74	3·07
1 after	9·95	1·83	4·93	8·60	2·96	4·62	10·30	2·23	6·08	7·65	2·87	5·67
2 ,,	9·45	2·25	4·66	8·35	2·45	4·35	10·70	2·00	5·58	8·70	2·26	4·82
3 ,,	9·25	2·00	5·07	8·95	2·02	4·82	10·80	1·87	6·89	8·45	1·90	4·82
4 ,,	10·50	2·72	4·72	9·35	1·51	5·19	12·05	1·78	7·26	10·30	1·82	5·58
5 ,,	10·55	2·48	5·87	9·85	1·80	5·11	11·40	1·60	6·75	10·40	2·00	5·53
6 ,,	10·85	2·38	5·92	10·55	1·84	5·53	10·75	1·60	6·75	9·70	2·01	5·40
	Cow 11			Cow 12			Cow 13			Cow 15		
2 before	11·05	2·17	6·08	9·65	2·61	9·00	10·25	—	4·85	—	—	—
1 ,,	8·85	2·48	4·44	10·75	3·22	8·04	10·35	1·60	4·50	9·60	2·26	6·13
Calving	6·10	2·81	2·66	7·95	—	4·82	8·25	1·85	2·81	7·80	2·58	6·13
1 after	6·25	2·96	3·24	9·25	3·69	5·40	9·50	2·45	5·19	6·35	3·57	4·93
2 ,,	7·85	2·78	4·47	9·35	2·95	5·87	9·30	2·20	5·15	—	—	6·08
3 ,,	11·30	1·35	6·03	9·70	2·14	5·58	10·15	2·19	5·58	5·95	2·96	3·82
4 ,,	11·50	1·82	7·10	10·80	1·97	5·19	10·60	1·86	6·08	6·75	2·74	3·52
5 ,,	11·25	1·87	8·44	10·15	1·47	5·67	9·40	1·80	6·49	6·50	2·37	2·70
6 ,,	11·05	2·12	6·82	10·20	2·25	6·19	9·50	1·99	5·04	9·45	0·94	3·92
	Cow 16			Cow 18			Cow 19			Cow 20		
2 before	10·55	2·25	5·67	11·10	2·78	5·58	10·65	2·74	4·27	12·15	2·12	5·11
1 ,,	9·80	2·45	4·51	7·20	3·81	3·75	12·40	2·48	4·25	11·55	2·17	4·32
Calving	9·30	2·00	5·82	7·35	4·05	3·84	9·65	2·85	3·09	8·75	2·74	2·78
1 after	10·25	2·00	5·23	10·05	3·63	5·62	10·90	2·15	5·15	11·05	2·61	5·77
2 ,,	10·05	2·26	5·02	11·40	2·02	5·67	10·75	2·23	4·21	11·10	1·82	5·82
3 ,,	10·05	2·17	4·59	11·80	1·81	6·85	9·80	2·19	4·40	10·95	1·67	6·03
4 ,,	10·95	2·00	4·56	11·35	1·81	6·79	10·50	1·82	6·03	10·00	1·97	4·38
5 ,,	10·65	1·94	4·82	13·50	1·89	5·84	9·70	1·99	6·10	10·45	1·77	4·38
6 ,,	11·15	2·01	5·11	11·85	2·00	5·40	10·05	2·23	6·03	11·20	1·72	5·11

Table II. *Composition of the blood of 8 calves from birth to 8 weeks.*

All values in mg. per 100 ml.

Calf No.		Birth	2 days	4 days	6 days	14 days	21 days	28 days	35 days	42 days	49 days	56 days
4	Serum-Ca	13·85	14·25	13·20	12·90	12·35	12·35	12·75	11·95	11·35	11·40	11·90
	Serum-Mg	2·10	1·58	1·70	1·82	1·83	2·20	1·99	1·88	1·93	1·58	1·60
	Blood-inorganic P	6·43	6·08	5·87	4·78	6·37	5·72	6·61	6·65	6·23	6·46	6·31
5	Serum-Ca	13·55	13·45	13·45	14·40	12·95	12·95	12·80	13·60	13·10	13·10	12·95
	Serum-Mg	1·95	1·43	1·43	1·78	1·42	1·74	1·97	1·97	2·21	1·80	1·81
	Blood-inorganic P	7·34	6·08	6·82	7·58	8·34	7·10	7·42	7·58	6·40	8·14	7·34
7	Serum-Ca	14·00	14·15	13·85	12·80	12·35	12·40	13·35	12·65	12·30	13·95	12·60
	Serum-Mg	1·74	1·12	1·69	1·55	1·83	2·00	2·12	2·05	2·20	2·25	2·12
	Blood-inorganic P	6·55	6·19	6·08	7·10	7·18	7·94	7·84	8·44	7·42	6·43	6·68
8	Serum-Ca	12·85	13·30	—	12·75	11·75	13·45	12·35	12·55	12·75	12·05	11·55
	Serum-Mg	1·63	1·58	1·78	1·74	1·80	1·80	1·81	1·89	1·79	2·05	1·92
	Blood-inorganic P	4·35	5·19	6·55	6·13	6·25	6·82	6·75	5·97	6·37	6·55	6·70
11	Serum-Ca	13·20	13·15	12·45	12·25	13·00	13·30	12·55	14·05	12·80	13·50	12·30
	Serum-Mg	1·82	1·81	1·51	1·66	2·00	2·00	1·94	2·32	2·24	2·44	2·16
	Blood-inorganic P	8·24	6·08	5·62	6·03	5·62	7·68	6·82	7·18	7·26	6·89	7·46
12	Serum-Ca	12·70	14·15	14·45	14·20	12·45	11·75	12·05	13·10	12·55	12·05	13·75
	Serum-Mg	—	1·58	1·81	2·04	1·86	2·12	2·00	2·12	2·25	2·50	2·37
	Blood-inorganic P	4·47	6·19	6·37	6·13	6·37	6·37	5·87	6·43	8·24	7·58	7·54
13	Serum-Ca	12·95	12·05	13·80	12·60	12·00	11·25	11·95	12·15	11·90	13·90	12·25
	Serum-Mg	1·21	1·50	1·37	1·86	1·39	2·25	1·86	1·47	1·70	1·86	1·64
	Blood-inorganic P	6·37	5·15	7·50	6·82	6·75	7·10	6·03	6·75	6·25	6·75	6·55
15	Serum-Ca	13·70	13·05	13·20	13·75	12·50	12·25	12·75	13·05	12·30	12·65	11·90
	Serum-Mg	1·72	1·40	1·71	1·55	1·49	1·72	1·84	1·47	1·90	2·00	1·69
	Blood-inorganic P	7·50	5·62	6·55	7·50	6·82	6·08	5·67	6·13	4·47	6·68	6·43

studied for a longer period of time, namely 8 weeks. The method used for serum-Mg was a modification of that of Denis [1922] in which the phosphorus in the magnesium ammonium phosphate precipitate obtained from the Ca-free serum was estimated by the colorimetric method of Fiske and Subbarow [1925].

The data for each cow are given in Table I and curves of average values (excluding Cow No. 15) are given in Fig. 1. The data for 8 calves are given in Table II and the curves of average values in Fig. 2.

Discussion of results.

It should be noted that the basal ration of the whole of the cows was considered to be low in Ca. Of the cows, Nos. 5, 8, 12, 13, 15 and 18 received only this ration, whilst Nos. 4, 7, 11, 16, 19 and 20 received, in addition, a supplement of Ca salts to balance the ration. Whilst there are fairly wide variations between individual cows, these are not due to the dietary difference between the two groups, as may be seen by a study of the data in Table I.

As before, the tendency is for a fall in serum-Ca and blood-inorganic P just prior to parturition, with the lowest points at the time of calving: the only exception to this was Cow No. 16 in which the inorganic P was higher at calving. The labour of this cow was difficult and delayed and it is possible that true labour commenced 24 hours earlier, at the time of the lowest value for inorganic P. Thus we have confirmed our previous findings for the changes in serum-Ca and blood-inorganic P.

As mentioned above the data from Cow No. 15 were excluded from the averages. This was due to the fact that she suffered from a mild attack of milk fever which is reflected in the time taken for the serum-Ca to rise to its normal level and a corresponding high level of serum-Mg during that period.

Considering the results as a whole the behaviour of serum-Mg is more erratic than is that of serum-Ca or blood-inorganic P and the results are consequently less easy to assess. The curve in Fig. 1 reveals a tendency for the level of serum-Mg to be raised just at or for 24 to 48 hours after calving, but in some individual cows this rise occurred the day before calving. The post parturient fluctuations are similarly relatively wide. On the whole there appears to be a tendency for serum-Ca and serum-Mg to vary inversely.

In the case of the calves the data again showed a higher initial level of serum-Ca and blood-inorganic P than those normal for the dam. The serum-Ca dropped after 14 days from a level of 13·5–13·8 down to a level of about 12·4 and thereafter remained steady between 12·3 and 12·8, this being true for every calf except No. 11. Serum-Mg at birth was definitely of a lower order than that of the dam's normal and remained so for the first 14 days. After 21 days it had attained and remained fairly constant around the normal level for the cow.

Summary.

Earlier data on the decrease in serum-Ca and blood-inorganic P of cows just before and at calving are confirmed and data presented to show that, while the serum-Mg fluctuates rather widely about this period, it tends to rise to a higher level just before, at, or within 24 hours of calving. In general serum-Mg varies inversely with serum-Ca. Calves at birth and for the first 8 weeks of life show rather higher levels of serum-Ca and blood-inorganic P than the normals for

the dam, while the serum-Mg level at birth is lower than the dam's normal, but has attained this normal after 3 weeks.

We wish to acknowledge our indebtedness to Mr W. Thomson for assistance with the animals.

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CXLI. THE SALTING-OUT OF AMINO-ACIDS FROM PROTEIN HYDROLYSATES.

I. THE ISOLATION OF TYROSINE, LEUCINE AND METHIONINE.

By EVELYN MARY HILL and WILLIAM ROBSON.

From the Physiology Department, University of London King's College.

(*Received May 4th, 1934.*)

Requiring a supply of *l*-methionine for experiments designed for an attempt to throw light on the intermediate metabolism of this amino-acid, we were beset at the outset of our work with the problem of obtaining it in quantity. The useful and interesting paper by Pirie [1932] on its isolation from caseinogen hydrolysates had not then been published, and the only method which was likely to give us the amount deemed necessary for our purpose was that of synthesis. A quantity of the racemic acid was actually synthesised on the lines described by Barger and Weichselbaum [1931] with satisfactory results. It was soon realised, however, that resolution would involve so much material and time that we were obliged to turn to the reconsideration of Mueller's [1923] original method of isolating the active amino-acid and attempt to improve his yields.

At this stage of our investigation Pirie's paper appeared and we were able by his method to isolate a quantity of the active amino-acid. The serious difficulty in the procedure, as the author himself indicates, lies in the butyl alcohol extraction of the acid hydrolysate, both layers being so darkly coloured that at times it is impossible to distinguish the interface. It appears to us, moreover, that the method adopted for removing mercury from the mercury-methionine precipitate by heating the latter with a solution of barium hydroxide is liable to lead to loss of amino-acid. Mercuric oxide in hot alkaline solution is an excellent reagent for the rapid oxidation of amino-acids; we have avoided its use by decomposing the precipitate with hydrogen sulphide. It must be added, however, that Pirie [1933] has since described a second method which avoids the extraction by butyl alcohol. Of this we have had no experience.

In repeating Pirie's [1932] procedure a second time, we sought some means of purifying the acid hydrolysate before the butyl alcohol extraction. Pirie himself used lead acetate with only partial success. After several unsuccessful attempts with various reagents, we finally obtained a transparent and only slightly coloured solution by boiling the hydrolysate with a good grade of charcoal, the p_H of the solution having previously been raised to 4·5 by the addition of sodium hydroxide. On the occasion when this was done, the solution had to remain overnight before the extraction by butyl alcohol could be carried out. Next morning there had separated a copious crystalline precipitate which, as might be expected, consisted mainly of tyrosine. Leucine was also present, but to our great interest, the mixture, after alkaline fusion, gave an intense nitroprusside reaction. That this reaction was not due to cysteine was proved by boiling a portion of the solid with a solution of sodium plumbate, when no precipitate of lead sulphide was obtained. The probability that cystine is not present in the precipitate receives support from other directions. Pfeiffer and

Angern [1924], for example, showed that whilst ammonium sulphate can precipitate cystine from aqueous solution, sodium chloride will not do so; again, Okabe [1928] has found that the solubility of cystine in sodium chloride solution is considerably greater than in distilled water. Ruling cystine out in this way, it was apparent that the nitroprusside reaction was due to methionine, and it became clear that, if the above tyrosine complex contained methionine in any quantity, the problem of the isolation of the latter amino-acid would be considerably simplified.

The investigation was proceeding on these lines when our attention was drawn to a paper by Barnett [1933] on the salting-out of leucine from protein hydrolysates. It will be seen from Barnett's curve (Fig. 1) that the minimum solubility of leucine in saturated sodium chloride solution lies at p_H 2·4, at which point, as the author shows, the amino-acid is precipitated as dileucine hydrochloride. In studying Barnett's paper it appeared to us that methionine might reasonably be expected to be precipitated with leucine from saturated salt solution at p_H 2·4. Subjecting a caseinogen hydrolysate to Barnett's procedure, we found that methionine was actually present in the precipitate obtained at p_H 2·4, and that Barnett had apparently overlooked its presence therein. Unfortunately from our point of view, a sulphur determination carried out on the precipitate showed us that methionine was present in only very small amount.

Finding that Barnett's leucine fraction would not yield us methionine in promising yield, we turned to another aspect of this salting-out phenomenon. We have included in Fig. 1 a curve showing the results obtained by Sano [1926] in an investigation of the solubility of leucine in aqueous solution at different values of p_H. Comparison of the curves of Barnett and Sano shows that the effect of sodium chloride in reducing the solubility of leucine is at a maximum at p_H 2·4 and almost as great in the neighbourhood of p_H 6, *i.e.* at the isoelectric point of the amino-acid. From a knowledge of their chemical constitutions it can safely be assumed that the isoelectric points of leucine and methionine cannot be far removed from one another. The question remained whether the yield of methionine would be increased if the protein hydrolysate, after adjustment to p_H 6, were saturated with sodium chloride. An experiment based on this reasoning appeared worth undertaking.

The tyrosine-leucine-methionine mixture which had been precipitated after decolorising the protein hydrolysate at p_H 4·5 was dissolved in the minimum quantity of water and kept overnight. Next morning the tyrosine had separated in exceptionally good quality and yield. After filtration, the mother-liquor containing some leucine and methionine was added to the original bulk of the hydrolysate. The combined solutions, the p_H of which had been adjusted to 6 by the addition of sodium hydroxide, were then concentrated *in vacuo*. Ultimately crystals of sodium chloride began to separate; the evaporation was then stopped and the mixture placed in the ice-chest overnight. During this time the separated salt had settled to the bottom, whilst floating near the surface was a crystalline mass consisting, as shown by examination, chiefly of leucine and methionine. By decanting the solution from the salt, the separated amino-acids were collected, redissolved in water, and the methionine isolated according to Pirie's procedure. After a second precipitation, the yield of methionine, which, from sulphur determinations, was found to be of a high degree of purity, only amounted to some 0·6 % of the caseinogen used. Examination of the leucine-methionine solution from which the methionine had been precipitated showed that there still remained dissolved some of this amino-acid which had escaped precipitation by mercuric acetate. It was clearly evident that the conditions

for precipitating most of the methionine from the solution were not ideal for the purpose.

A series of semi-quantitative experiments on the following lines proved that this was not only true, but that it would probably be a matter of some difficulty, owing to the presence of other amino-acids, to regulate the conditions so that

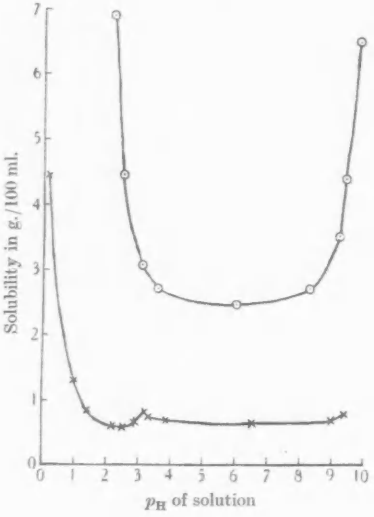

Fig. 1.

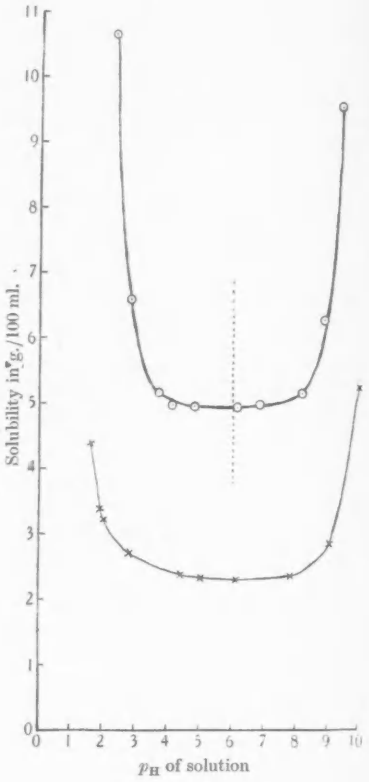

Fig. 2.

Fig. 1. ⊙—⊙ Solubility of leucine in water (Sano); ×—× Solubility of leucine in saturated sodium chloride solution (Barnett).

Fig. 2. ⊙—⊙ Solubility of methionine in water; ×—× Solubility of methionine in saturated NaCl solution.

most of the methionine would be precipitated. If, for example, to a solution of leucine, a solution of mercuric acetate in acetic acid be added drop by drop, a definite precipitation occurs. This precipitate however dissolves on the addition of (1) further mercuric acetate solution, or of (2) sodium chloride solution. Similar experiments with a solution of methionine showed that the precipitate produced by the addition of the mercuric acetate solution was soluble only in a

very much greater excess of the latter reagent, and that, while the presence of sodium chloride certainly favours the complete precipitation of the methionine, if the concentration of the sodium chloride exceeds a certain value, re-dissolution of the mercury-methionine precipitate occurs. Experimentation on these lines is being continued and will be reported in an early communication. At the present time it will suffice to add that if, after the precipitate obtained by treating the above leucine-methionine solution with mercuric acetate reagent has settled, sodium chloride solution be carefully added, a further precipitation takes place with the result that the ultimate yield of methionine obtained is raised from the above figure of 0·6 % to one of 1·5–1·6 %. Such yields have been obtained from caseinogen on several occasions. Egg-albumin, subjected to the same procedure, has given yields averaging 2·5 %.

The leucine was finally obtained by treating the solution, from which the mercury-methionine compound had been filtered, with hydrogen sulphide, filtering off the precipitated mercuric sulphide, concentrating the filtrate *in vacuo* to a syrup, dissolving the latter in the minimum quantity of water and raising the p_H to 6, when the leucine crystallises as the solution cools. Recrystallisation of the amino-acid follows in the usual way.

It was considered of general interest to include in this communication curves showing the solubility of methionine in aqueous and saturated sodium chloride solutions and these are shown in Fig. 2. The upper curve (solubility of methionine in aqueous solutions) appears to be symmetrical about an axis which cuts the p_H abscissa in the close neighbourhood of 6, a figure which may be taken as a close approximation to the isoelectric point of methionine. It is noteworthy moreover that the curve showing the solubility of methionine in saturated sodium chloride solution does not exhibit that dissymmetry which is seen in the corresponding curve for leucine (Fig. 1).

The average value of several actual yields of recrystallised amino-acids obtained from caseinogen (ash content 1·1 %, moisture 9 %, N 15 %) and egg-albumin (ash content 4·9 %, moisture 7·4 %, N 15·2 %) are as follows:

	Caseinogen %	Egg-albumin %
Tyrosine	3·0	1·35
Leucine	4·7	6·1
Methionine	1·55	2·5

Experimental.

1 kg. of caseinogen was hydrolysed with 2 litres of 20 % hydrochloric acid for 18–20 hours. The excess hydrochloric acid was removed in the usual way by concentration *in vacuo*, the residue being redissolved in hot water and evaporated until viscous.

Separation of tyrosine. The final residue was dissolved in 4 litres of boiling water, the solution treated with sufficient 40 % sodium hydroxide solution (approx. 460 ml.) to bring the p_H to 2·4, 40 g. of charcoal added, and the mixture, after being boiled for 20–30 minutes, was filtered hot. The charcoal-humin residue was extracted with about 1 litre of boiling water, and the filtered extract added to the main solution. The combined solution, which was quite clear and only faintly coloured, was allowed to cool overnight when the tyrosine crystallised out. This was filtered off and the filtrate used for the isolation of the methionine and leucine as described below. To increase the purity of the tyrosine thus obtained, it was recrystallised from boiling water in the usual way.

Separation of methionine. To the mother-liquor from the tyrosine, sodium hydroxide solution was cautiously added to p_H 6·0, and the resulting solution was evaporated under reduced pressure until sodium chloride commenced to separate (at approximately 1800 ml.). After cooling, the sodium chloride had settled to the bottom and the separated amino-acids were mainly floating near the surface of the liquid. The mother-liquor and the amino-acids were decanted to a Büchner funnel, and the acids sucked as dry as possible. The sodium chloride remaining in the container was separately dissolved in the minimum amount of water, and the solution thus obtained was used to wash the amino-acids on the Büchner free from mother-liquor.

The crude mixture of solid leucine and methionine obtained in this way was dissolved in 2 litres of water, and a cold saturated solution of mercuric acetate in 3 % acetic acid was added until no further precipitation occurred (about 250 g. mercuric acetate). After keeping for some time, a saturated solution containing approximately 60 g. of sodium chloride was added, when a further heavy precipitate formed. The mixture was kept overnight, and the mercury precipitate was then filtered off, suspended in about 2 litres of warm water and decomposed with hydrogen sulphide. The mercuric sulphide was boiled out with water, the mixture filtered and the filtrate added to the main methionine solution.

The solution containing the crude methionine was now evaporated almost to dryness, the residue dissolved in some 200 ml. of alcohol, the p_H of the alcoholic solution adjusted to 6·0 by the addition of pyridine and the mixture left in the ice-chest overnight. Next morning, the precipitate of crude methionine was filtered off, washed with alcohol to remove any pyridine and finally dried with ether.

For purification the product was redissolved in boiling water (1 litre for every 10 g. of crude methionine), a boiling saturated solution of mercuric chloride (10 g. of mercuric chloride per g. of the amino-acid) added, and the mixture, after being boiled for some minutes, was set aside until cold. The mercury precipitate then formed a solid mass, from which the mother-liquor was easily decanted. The solid was transferred to a mortar, ground up with water, the mixture filtered and the recovered solid suspended in warm water and decomposed with hydrogen sulphide. The further stages of the separation of the methionine followed the lines described above.

A further recrystallisation may be carried out from 75 % alcohol; it is inadvisable to use water since the solubility of methionine in this medium is approximately 5 % (Fig. 2). If, as sometimes happens, it is found difficult to remove the last traces of pyridine from the methionine, recrystallisation from 75 % alcohol, to which a few drops of acetic acid have been added, will enable this to be done.

Separation of leucine. The solution, from which the methionine was first precipitated by mercuric acetate, was freed from mercury by treatment with hydrogen sulphide. The mercuric sulphide was filtered off, the sulphide washed with boiling water, and the combined filtrates were concentrated *in vacuo* to a small bulk. The concentrate was dissolved in a small quantity of water and the p_H adjusted to 6·0, when the leucine at once began to crystallise. After some hours, the separated leucine was filtered off and recrystallised from dilute alcohol. This completed the procedure.

Summary.

A simple procedure is described for the isolation from caseinogen hydrolysates of tyrosine, methionine and leucine. The yields, especially those of tyrosine and methionine, obtained compare very favourably with figures already in the literature for these amino-acids.

The work forming the basis of this paper was done during the tenure by one of us (E. M. H.) of a Berridge Studentship awarded by the Delegacy of the University of London King's College.

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CXLII. AN EFFECT OF ASCORBIC ACID ON FATTY ACID OXIDATIONS IN THE LIVER.

By JUDA HIRSCH QUASTEL and
ARNOLD HERBERT MAURICE WHEATLEY.

From the Biochemical Laboratory, Cardiff City Mental Hospital.

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In the course of experiments on the oxidation of fatty acids by the liver of guinea-pigs it was found that the yield of acetoacetic acid appeared to depend on the state of nutrition of the animal. Preliminary experiments indicated that the livers of guinea-pigs fed for a lengthy period on a scorbutic diet behaved differently from the normal, a lower yield of acetoacetic acid being produced, and for this reason work was commenced on the effects of ascorbic acid on fatty acid oxidations of liver. Although further experiment has not shown a very strict line of demarcation between the activities of the livers of scorbutic and normal guinea-pigs, it has become abundantly clear that ascorbic acid exercises very definite effects on the respiration of, and on the power to oxidise fatty acids by, the livers of both guinea-pigs and rats.

It appears that the most significant effect of the addition of ascorbic acid to liver slices is the maintenance of respiration at a relatively high level for a much longer period than occurs in liver slices to which ascorbic acid has not been added. This is particularly noticeable with the rat where the respiration of a liver slice falls off quickly after an initial period of a fairly constant rate of oxygen uptake.

It is much less noticeable in the guinea-pig where the Q_{O_2} tends to remain constant over a long period; hence results with guinea-pigs are irregular and apparently much more dependent on nutritional conditions than is the case with the rat. Increases of acetoacetic acid production ranging from 30 to 200 % due to the addition of ascorbic acid have been noted with the rat and from 0 to 60 % with the guinea-pig; these increases in the yield of acetoacetic acid are probably closely connected with the maintenance of the relatively high levels in the rate of oxygen uptake, the oxidation of fatty acids being greatly dependent on the general respiratory metabolism of the liver cell.

Technique.

The technique described by the writers [1933] in their work on the oxidation of fatty acids in the liver has been followed. Measurements of oxygen uptake and acetoacetic acid yield (at 37°) have been obtained with the Barcroft differential manometer. Acetoacetic acid has been estimated manometrically by the method described by the writers [1933]. A small change in the technique of this estimation has been adopted: 1 ml. $M/5$ acetate buffer p_H 3·8 has been replaced by 1 ml. $M/2$ acetic acid. The results are equally accurate and consistent.

The acetoacetic yield is expressed by the symbol $Q_{\text{acetoacetic acid}}$ or Q_{ac} which represents the μl. CO_2 equivalent to acetoacetic acid produced by 1 mg. dry weight liver tissue per hour.

The only animals used in this work have been guinea-pigs (from various sources) and rats (also from various sources). We have recently confined ourselves, in work with rats, to the Wistar strain and find that this procedure is more satisfactory for obtaining comparable results.

The substrates under investigation have been butyric, crotonic and *dl*-β-hydroxybutyric acids. These have been chosen because they are very good sources of acetoacetic acid and also because the mechanism of their oxidation to acetoacetic acid is at present under investigation in this laboratory. A few experiments with higher fatty acids such as *n*-hexanoic acid have also been carried out and found to give results essentially similar to those found with butyric and crotonic acids.

Buffer solution. It was shown by the writers [1933] that concentrations of phosphate buffer (p_H 7·4) of the order $M/15$ appreciably diminish both Q_{O_2} and Q_{ac} of guinea-pig liver, and this was traced to the removal of calcium ions from the medium bathing the liver slices. Low concentrations of phosphate buffer solution do not buffer well in experiments lasting 3 hours or more. The difficulty of adequate buffering has been overcome (or partially so) without affecting the concentration of calcium ions by the use of sodium glycerophosphate buffer. Sodium glycerophosphate (B.D.H.) solution was brought to p_H 7·4 by addition of hydrochloric acid, and this was diluted by Locke solution and the substrate under investigation to make its final concentration $M/15$. Experience has shown that with both guinea-pig and rat liver slices of the order of 20 mg. dry weight there is a drop in p_H, in an experiment of 3 hours' duration, of about 0·2.

The final medium was made up as follows:

1·0 ml. $M/5$ sodium glycerophosphate solution (p_H 7·4).
1·5 ml. Locke solution[1].
0·5 ml. 0·1 M substrate solution.

Such a glycerophosphate-Locke medium has been used in the experiments described below.

Liver slices were added to this medium, and then 0·5 ml. of a neutral solution of ascorbic acid was added. All experiments with liver slices were carried out in an atmosphere of oxygen.

The manometric apparatus was allowed to shake at the rate of 90 times a minute, and readings were taken every 15 minutes.

After 3 hours, the liver slices were carefully removed, washed in distilled water and dried at 105°. Usually very little disintegration of the liver slice occurred. 2·5 or 3 ml. of the medium were then transferred to another Barcroft apparatus for estimation of acetoacetic acid. Determinations of final p_H were always made, and the results of any experiment where there had been a considerable change in p_H were discarded.

Choice of liver slices. It is important for the investigation of fatty acid oxidation with liver slices to select slices of appropriate sizes and thicknesses. It has been shown by the writers [1933] that mincing liver results in a cessation of fatty acid oxidation and it would be expected that very thin slices of liver

[1] Occasionally we found it desirable to use only 1 ml. Locke solution. Care was always taken, however, to use the same quantity of Locke solution in all vessels in any one experiment. The Locke solution was composed of 0·003 M $CaCl_2$, 0·0065 M KCl and 0·1445 M NaCl.

would approximate to the condition of minced tissue. Experience shows that liver slices less than 0·2 mm. thick give irregular results; the Q_{O_2} usually falls off rapidly and there is a diminished Q_{ac}. There is also an upper limit to the thickness of the slices used, determined probably by the diffusion of oxygen. Slices thicker than 0·4 mm. give a diminished Q_{O_2} and Q_{ac}. A few descriptive results are shown in Table I.

Table I. *Effects of varying thicknesses of liver slices (guinea-pig) on* $Q_{acetoacetic\ acid}$ *in presence of crotonate, 0·017* M. *(Glycerophosphate-Locke medium.)*

Exp.	Thickness mm.	Q_{ac}
1	0·28	2·3
	0·43	2·4
	0·56	1·6
2	0·29	2·0
	0·27	2·0
	0·67	1·1

We have therefore tried in all experiments to cut slices varying between 0·2 and 0·4 mm. Moreover in any one experiment where comparisons are made on one tissue it has been our endeavour to use slices of approximately the same thickness. Rat liver is more difficult to cut than guinea-pig liver and it is usually not an easy matter to obtain a large slice of uniform thickness. Hence we have used two or three small slices of approximately uniform thickness (as determined superficially by opacity or colour) rather than one large slice. Slices are always trimmed—a slice with frayed edges tends to disintegrate in the manometer vessel and to give, therefore, too low a weight after removal at the termination of the experiment.

The thickness of the slices is determined by floating them in a Petri dish containing saline, on the bottom of which is a sheet of squared paper. The area of the slice is outlined on the paper as carefully as possible and the area calculated in mm.^{2} With trimmed, rectangular slices the area can be obtained in this way with considerable accuracy. The thickness of a slice can now be calculated from the volume ($=5\times$ dry wt. in mg.) and the area (mm.^{2}). With experience, slices of thicknesses varying from 0·2 to 0·4 mm. can be chosen with ease.

The livers are cut immediately after removal from an animal which has been killed by cutting the throat and then well bled.

Source of ascorbic acid. Two samples of ascorbic acid have been used in the work; one was kindly presented to us by Prof. A. Szent-Györgyi. The other was kindly given to us by the Hoffmann-La Roche Co. according to whom the analytical figures were: $[\alpha]_D = +24°$; M.P. 188–189°; equiv. wt. (iodine titration) $=88$. The two samples behaved in the same way.

Differences between guinea-pig liver and rat liver.

Representative results indicating the respiration of, and acetoacetic acid production by, guinea-pig and rat livers are shown in Table II. Detailed results concerning guinea-pig liver oxidations are given in our earlier paper [1933] and will not be further referred to here. The most striking difference between rat and guinea-pig livers is the high Q_{O_2} of the former compared with that of the latter. Rat liver usually gives a Q_{O_2} of 10–12; guinea-pig liver gives a Q_{O_2} of 4–6.

Another difference, which will be commented upon again later, is that whilst the oxygen uptake of guinea-pig liver (under the experimental conditions

Table II. *Respiration of, and acetoacetic acid production by, liver slices from guinea-pig and rat.*

Substrates present as Na salts.

Exp.	Substrate (0·017 *M*)	Q_{O_2}	Q_{ac}
1. Guinea-pig liver	Nil	4·0	0·2
,,	Crotonate	9·0	3·6
,,	Butyrate	10·5	4·4
,,	*dl*-β-Hydroxybutyrate	6·1	1·8
2. Rat liver	Nil	11·6	0·8
,,	Crotonate	12·8	4·9
3. Rat liver	Nil	10·7	0·9
,,	Butyrate	12·6	3·0
4. Rat liver	Nil	9·5	0·7
,,	*dl*-β-Hydroxybutyrate	11·9	6·4

already described) is usually constant for 2 or 3 hours, that of rat liver begins to fall off rapidly after a preliminary period of fairly constant oxygen uptake which varies from 45 to 90 mins. As a general rule the thinner the slice the quicker is the tendency for the oxygen uptake to fall from a linear rate.

When sodium butyrate or sodium crotonate at a concentration of 0·014 *M* is added to guinea-pig liver there is a decided increase in Q_{O_2}, the figures in Table II being quite typical. The rise in Q_{O_2} with rat liver due to butyrate or crotonate is always very much less and we have had cases when the Q_{O_2} of rat liver has not been changed by the addition of butyrate or crotonate, although it was clear from the acetoacetic acid production that oxidation of the fatty acid had been proceeding.

The acetoacetic acid production from guinea-pig liver in presence of butyrate or crotonate is variable and seems to be dependent on the size of the liver. As a general rule the larger the liver in a well fed animal the smaller is the yield of acetoacetic acid. This observation will be commented upon at greater length in another communication. The acetoacetic acid production from *dl*-β-hydroxybutyric acid (0·014 *M*) with guinea-pig liver is much less variable than that from butyric or crotonic acid. With this animal the Q_{ac} is usually of the order 1·8 whilst that from butyrate and crotonate may vary from 1·5 to 4·5. With rat liver the yield of acetoacetic acid from *dl*-β-hydroxybutyric acid is considerably higher than with guinea-pig and the Q_{ac} is of the order 4–6. The Q_{ac} with rat liver from butyrate and crotonate is lower and has about the same range of variation as with guinea-pig.

We have observed that the rate of oxygen uptake tends to fall off to a greater extent in the presence of crotonate than in the presence of butyrate, and this seems to apply to both guinea-pig and rat.

Oxidation of ascorbic acid in the liver.

Harrison [1933] in his experiments on the effect of ascorbic acid on the respiration of scorbutic and normal guinea-pig tissue "balances" the ascorbic acid in the manometric vessels by placing the acid in phosphate buffer without tissue in the left-hand vessel of the Barcroft apparatus. This we have not done in our experiments because there is no proof that the rate of oxidation of ascorbic acid in the presence of tissue is equal to or is of the same order as that in the absence of tissue. This constitutes only a minor criticism of Harrison's results, for he used smaller quantities of ascorbic acid than we have used.

The following experiments indicate, with intact liver slices, that after 3 hours at 37° ascorbic acid is by no means fully oxidised, although it is so in the absence of tissue.

Exp. 1. 0·5 ml. of a neutral solution of ascorbic acid containing approximately 3 mg. per ml. was added to 3 ml. glycerophosphate-Locke medium and allowed to autoxidise in the presence of oxygen under the same conditions as were used for work with liver slices. After 3 hours 1 ml. of the medium was added to 4 ml. 3 % trichloroacetic acid and titrated with $N/200$ iodine solution. 0·04 ml. was required. The ascorbic acid was therefore practically completely oxidised under the given experimental conditions.

Exp. 2. 0·5 ml. of a neutral solution of ascorbic acid containing approximately 3 mg. per ml. was added to a glycerophosphate-Locke medium containing rat liver slices and to a glycerophosphate-Locke-crotonate (0·014 *M*) medium containing rat liver slices. After 3 hours' shaking in oxygen at 37°, the slices were removed and 1 ml. of each of the media was added to 4 ml. 3 % trichloroacetic acid and the solution titrated with $N/200$ iodine solution. The same procedure was carried out in control experiments where no ascorbic acid had been added. The following iodine titres were obtained:

			$N/200$ iodine titre ml.
(*a*) Glycerophosphate-Locke medium	Liver slices	No ascorbic acid	0·16
(*b*) Glycerophosphate-Locke-crotonate medium	,,	,,	0·12
(*c*) Glycerophosphate-Locke medium	,,	Ascorbic acid present	0·48
(*d*) Glycerophosphate-Locke-crotonate medium	,,	,,	0·49

An iodine titration on the ascorbic acid itself showed that 1 ml. of the medium would have required 0·95 ml. $N/200$ iodine had there been no oxidation of ascorbic acid. Hence from (*a*) and (*c*) 32/95, *i.e.* 33 % of the original quantity of ascorbic acid still remains and from (*b*) and (*d*) 38 % remains. Another experiment carried out under similar conditions showed that 49 % of the original quantity of ascorbic acid still remained at the termination of the experiment.

These experiments prove either (1) that the oxidation of ascorbic acid is retarded by liver slices, or (2) that oxidised ascorbic acid is reduced by liver slices, an equilibrium being established.

The second conclusion is the more likely one in view of Szent-Györgyi's experiments on the reduction of oxidised hexuronic acid by animal tissues [1928].

The action of ascorbic acid on the respiration of, and acetoacetic acid production by, normal guinea-pig liver.

According to Harrison [1933] ascorbic acid has but little effect on the respiration of normal guinea-pig liver. The probability is that there is a small but variable increase if the criticism mentioned above is taken into consideration. Our own experiments bear out this conclusion. Table III shows the effects of the addition of ascorbic acid[1] on the Q_{O_2} and Q_{ac} of normal guinea-pig liver slices in the presence of sodium crotonate or butyrate. It will be observed that the Q_{O_2} is in all cases raised, but the amount of rise is only about that to be expected by the oxidation of part of the ascorbic acid added. The expected change in Q_{O_2} (taken over 3 hours) for liver slices of 20 mg. dry weight is a rise of about 1·6 with an initial concentration of ascorbic acid of 0·5 mg./ml. but it is difficult to be certain of this as estimations of the amounts of ascorbic acid remaining at the end of the experiments were not made. It is clear that no significant increase attributable to the action of the added ascorbic acid on the oxidative metabolism of liver was obtained. Exp. 258 showed definitely a higher value of Q_{O_2} in presence of ascorbic acid but it is to be noted that the Q_{O_2} of the liver in this experiment in

[1] The ascorbic acid was neutralised with NaOH just before being added to the Barcroft vessels.

Table III. *Effects of ascorbic acid on the respiration of, and acetoacetic acid production by, liver slices of normal guinea-pig in presence of butyrate and crotonate.*

Substrate concentration 0·014 *M*. Glycerophosphate-Locke medium. Initial p_H=7·4. Final p_H=usually 7·1–7·2. Each experiment refers to a single animal.

Exp.	Weight of liver slices taken (mg. dry wt.)	Substrate	mg. per ml. ascorbic acid added	Q_{O_2} (av. over 3 hours)	Q_{ac}
128	24·3	Crotonate	Nil	9·5	3·1
	21·2	,,	0·43	10·4	3·5
139	24·2	Butyrate	Nil	8·5	3·0
	27·0	,,	0·50	9·6	2·9
140	38·8	,,	Nil	10·6	3·5
	34·5	,,	0·50	12·1	4·3
141	22·5	Crotonate	Nil	9·0	3·2
	18·5	,,	0·50	9·6	3·4
256	17·4	,,	Nil	7·6	2·4
	17·6	,,	0·86	9·8	3·2
258	14·6	,,	Nil	7·0	2·3
	12·1	,,	0·86	10·6	3·6

the absence of ascorbic acid was lower than usual; this was due to the fact that the rate of oxygen uptake fell off more rapidly than commonly occurred. In the presence of ascorbic acid the rate of oxygen uptake was constant throughout most of the experiment (see Table VI).

It will be noted that in most cases the presence of ascorbic acid produced an increase in Q_{ac}. The increase is usually small, but occasionally, as in Exp. 258, of the order of 50 to 60 %.

The results given in Table III are typical of a large number of experiments with normal guinea-pigs and show that whilst the effect of added ascorbic acid on the whole is small, irregularities (*i.e.* significant increases in Q_{O_2} and Q_{ac}) may occur.

Ascorbic acid and scorbutic guinea-pig liver[1].

The livers of guinea-pigs fed on a scorbutic diet for 15 days or longer usually show rises of Q_{O_2} and Q_{ac} in the presence of ascorbic acid, which are significantly greater than the average rises found with normal guinea-pigs. The animals were killed when they showed definite signs of scurvy and after their weights had reached a maximum and fallen 10–15 % of the maximum value. There is less irregularity about the results with scorbutic guinea-pig livers and an increase

Table IV. *Effects of addition of ascorbic acid on respiration of, and acetoacetic acid production by, liver slices from guinea-pigs fed on a scorbutic diet for varying periods.*

Substrate, crotonate 0·014 *M*. Conditions as in Table III.

Exp.	Period of scorbutic diet days	Weight of liver slices taken mg. dry wt.	mg. per ml. ascorbic acid added	Q_{O_2} (av. over 3 hours)	Q_{ac}
260	12	21·5	Nil	9·4	2·7
		21·0	0·86	11·2	3·3
264	15	17·6	Nil	8·6	2·9
		16·6	0·86	12·3	4·1
131	17	23·8	Nil	8·5	2·9
		24·4	0·50	10·1	4·0

[1] We are much indebted to the authorities of the Veterinary Laboratory, Weybridge, for supplying us with guinea-pigs in various stages of scurvy.

in Q_{ac} has always been found. The results of Table IV are typical. The percentage increase in Q_{ac} due to the addition of ascorbic acid is found to be always greater than the percentage increase in Q_{O_2}. It is worthy of note, however, that increases of the same order have been found occasionally with normal and apparently healthy guinea-pigs.

Ascorbic acid and normal rat liver.

Ascorbic acid has very definite effects on the Q_{O_2} and Q_{ac} of rat liver in the presence of sodium butyrate or sodium crotonate. The results given in Table V

Table V. *Effects of addition of ascorbic acid on the respiration of, and the production of acetoacetic acid by, normal rat liver in the presence of butyrate or crotonate.*

Substrate concentration 0·014 *M*. Conditions as in Table III.

Exp.	Weight of liver slices taken mg. dry wt.	Thickness of slices mm.	Substrate	mg. per ml. ascorbic acid added	Q_{O_2} (av. over 3 hours)	Q_{ac}
134	21·7	—	Butyrate	Nil	13·2	3·6
	25·5	—	,,	0·50	15·6	3·9
	17·2	—	Crotonate	Nil	10·0	2·5
	25·5	—	,,	0·50	14·3	4·4
160	22·8	—	,,	Nil	11·1	3·6
	20·5	—	,,	0·5	13·9	4·5
	18·3	—	,,	1·0	15·3	4·9
	19·7	—	,,	2·0	17·7	5·6
263	13·6	0·26	,,	Nil	12·2	3·1
	14·5	0·26	,,	0·86	13·0	4·3
266	14·0	0·27	,,	Nil	12·0	3·6
	12·8	0·26	,,	0·86	16·6	6·6
267	22·3	0·33	,,	Nil	12·4	3·2
	20·4	0·37	,,	0·86	13·2	4·5
268	16·6	0·30	Butyrate	Nil	14·1	3·2
	17·6	0·27	,,	0·86	16·5	4·5
	In the following experiments only Wistar rats were used.					
274	20·9	0·38	Nil	Nil	11·6	0·8
	19·6	0·37	Crotonate	Nil	12·9	4·9
	24·2	0·43	Nil	0·86	13·0	1·6
	20·2	0·39	Crotonate	0·86	16·7	8·4
275	13·6	0·24	Nil	Nil	8·0	0·5
	13·3	0·23	Butyrate	Nil	10·0	1·6
	16·5	0·25	Nil	0·86	11·2	1·0
	17·5	0·26	Butyrate	0·86	13·7	2·5
276	23·8	0·37	Nil	Nil	10·7	0·9
	22·4	0·33	Butyrate	Nil	12·6	2·9
	20·0	0·32	Nil	0·86	12·7	1·4
	18·3	0·30	Butyrate	0·86	17·8	5·3
283	15·9	0·28	Crotonate	Nil	9·4	1·8
	16·2	0·30	,,	0·43	15·6	4·1
	14·5	0·28	,,	0·86	15·1	4·5
	15·8	0·30	,,	1·3	14·7	5·1
285	20·3	0·22	Nil	Nil	8·4	0·3
	29·2	0·28	Crotonate	Nil	8·1	1·5
	20·7	0·23	Nil	0·43	12·2	0·4
	25·2	0·29	Crotonate	0·43	13·9	3·8
287	23·7	0·33	,,	Nil	11·3	2·2
	18·6	0·28	,,	0·43	15·1	2·6
288	26·8	0·33	,,	Nil	7·0	0·9
	20·0	0·30	,,	0·43	11·9	1·4

are typical of those found in about 50 experiments. The increases in Q_{O_2} and Q_{ac} are not regular and vary from rat to rat, but increases in Q_{ac} of the order of 80 % or more have often been found (see Exps. 134, 160, 266, 274, 276, 283, 285). Large increases in Q_{O_2} have also been obtained but as stated earlier these are more difficult to interpret owing to the fact that analyses of the ascorbic acid contents of the vessels at the termination of the experiments were not carried out. Rat liver usually gives a significant Q_{ac} in the absence of added substrates, and this value is invariably increased by the addition of ascorbic acid (see Exps. 274, 275, 276).

The problem now remains as to how ascorbic acid brings about the increased oxidation of butyric and crotonic acids—a very definite phenomenon in the case of rat liver and of scorbutic guinea-pig liver but doubtful, and at all events irregular, with normal guinea-pig liver. Control experiments have shown that the autoxidation of ascorbic acid does not induce oxidation of butyrate or crotonate (*i.e.* in the absence of tissue) and the negative results found with some guinea-pig livers confirm this.

It seems at first sight unlikely that ascorbic acid is essential for the oxidation of fatty acids, for scorbutic guinea-pig liver gives a Q_{ac} not far removed from normal. On the other hand no tests for ascorbic acid were carried out on the livers of the scorbutic animals and it is impossible for us to say what were the actual concentrations of ascorbic acid in the livers in question.

An explanation of this phenomenon, more satisfactory than one involving the assumption that ascorbic acid specifically affects fatty acid oxidation, comes from a consideration of the effects of ascorbic acid on the rates of oxygen uptake.

The effect of ascorbic acid on the rates of oxygen uptake by liver slices.

The rate of oxygen uptake by guinea-pig liver either alone or in the presence of crotonate and butyrate (0·014 *M*) is usually constant, the Q_{O_2} at the end of a 3 hours' experiment not being appreciably different from that half way through the experiment.

The presence of ascorbic acid does not alter the rate apart from a rise in Q_{O_2} at the commencement of an experiment owing probably to autoxidation of a portion of the ascorbic acid itself.

Rat livers, however, when slices are used of the thickness already quoted almost invariably show a fairly rapid fall in Q_{O_2} after an initial fairly steady rate of oxygen uptake, and at the end of 3 hours the Q_{O_2} may be less than 50 % of that half way through the experiment. The presence of ascorbic acid always diminishes this rate of fall, so much so that the rate of oxygen uptake may be the same at the end of 3 hours as it was at the end of $1\frac{1}{2}$ hours. In Table VI an analysis of most of the results already quoted in earlier tables is given.

Turning to the first part of the table dealing with guinea-pigs it will be seen that the percentage decrease in Q_{O_2} at the end of the experiment from that half way through is small and not appreciably affected by ascorbic acid. The percentage increase in Q_{ac} due to the ascorbic acid is also small, though appreciable in Exp. 256. In Exp. 258, there is a 17 % drop in Q_{O_2} which is removed by the presence of ascorbic acid; in this experiment the percentage increase in Q_{ac} is highest (50 %). There is little doubt from our experience that had these experiments been carried on for a longer period the percentage decrease in Q_{O_2} in the absence of ascorbic acid would have been greater and the percentage increase in Q_{ac} due to ascorbic acid considerably larger.

Table VI. *A comparison between the effects of ascorbic acid on acetoacetic acid production and the rate of fall of Q_{O_2} of liver slices.*

Exp.	Substrate	mg. per ml. ascorbic acid added	% increase in Q_{ac} due to added ascorbic acid	% decrease in Q_{O_2} at end of 3 hours from that at end of 1½ hours
		Guinea-pigs (normal).		
128	Crotonate	Nil	—	0
	,,	0·43	13	0
129	Butyrate	Nil	—	10
	,,	0·5	0	10
139	,,	Nil	—	0
	,,	0·5	0	10
140	,,	Nil	—	10
	,,	0·5	22	10
141	Crotonate	Nil	—	0
	,,	0·5	6	0
256	,,	Nil	—	23
	,,	0·86	33	20
258	,,	Nil	—	17
	,,	0·86	57	0
		Rats.		
263	,,	Nil	—	70
	,,	0·86	39	0
266	,,	Nil	—	40
	,,	0·86	83	8
267	,,	Nil	—	20
	,,	0·86	41	8
268	Butyrate	Nil	—	40
	,,	0·86	41	11
274	Crotonate	Nil	—	70
	,,	0·86	71	15
275	Butyrate	Nil	—	30
	,,	0·86	55	9
276	,,	Nil	—	37
	,,	0·86	81	9
283	Crotonate	Nil	—	66
	,,	0·43	126	10
	,,	0·86	150	11
	,,	1·3	188	9
285	,,	Nil	—	67
	,,	0·43	153	15

Turning to the second part of the table dealing with the rats we observe percentage decreases in Q_{O_2} varying from 20 to 70, these decreases being practically eliminated by the presence of ascorbic acid. It is in these experiments that the largest effects of ascorbic acid in increasing Q_{ac} are to be found.

Figs. 1–4 illustrate the action of ascorbic acid on the rate of change of Q_{O_2} of rat liver slices throughout an experiment. Fig. 1 shows the action of ascorbic acid on the change of Q_{O_2} with time of rat liver slices in a glycerophosphate-Locke medium containing no added substrate. Figs. 2 and 3 show the effects of differing quantities of ascorbic acid on the respiration of rat liver in presence of sodium crotonate. Fig. 4 refers to the effect of ascorbic acid on the change of Q_{O_2} of rat liver in presence of sodium butyrate. The following points are of note:

(*a*) There is an initial sharp fall in the rate of change of Q_{O_2} in the presence of ascorbic acid, the Q_{O_2} then arriving at a fairly constant value. The initial sharp fall is probably due to autoxidation of part of the ascorbic acid added.

(*b*) The constant Q_{O_2} found at the end of an experiment in which ascorbic acid has been added to the liver slices is somewhat less than, but usually not far removed from, the initial rates of oxygen uptake found with those liver slices to which ascorbic acid has not been added.

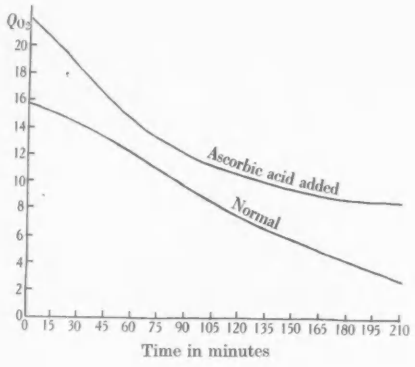

Fig. 1.

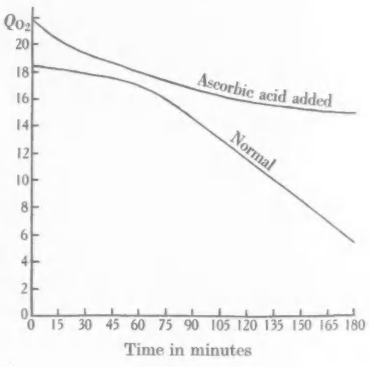

Fig. 2.

Fig. 1. Effect of addition of ascorbic acid (0·43 mg./ml.) on oxygen uptake by normal rat liver slices.

Fig. 2. Effect of addition of ascorbic acid (0·86 mg./ml.) on oxygen uptake of rat liver slices in presence of sodium crotonate (0·014 *M*).

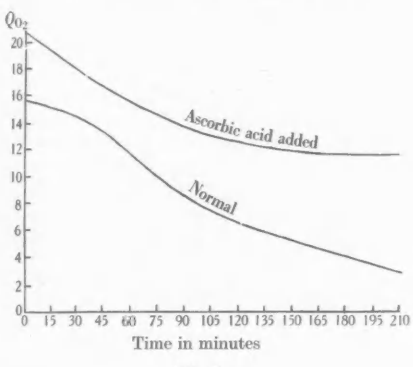

Fig. 3.

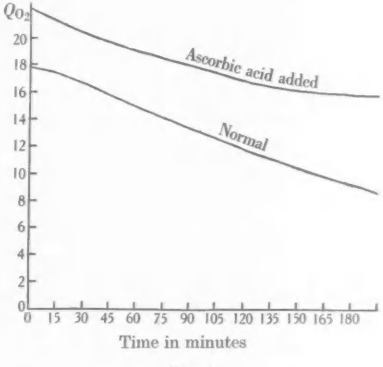

Fig. 4.

Fig. 3. Effect of addition of ascorbic acid (0·43 mg./ml.) on oxygen uptake of rat liver slices in presence of sodium crotonate (0·014 *M*).

Fig. 4. Effect of addition of ascorbic acid (0·86 mg./ml.) on oxygen uptake by rat liver slices in presence of sodium butyrate (0·014 *M*).

(*c*) Essentially the same phenomenon—a maintenance of a relatively high rate of respiration induced by the added ascorbic acid—occurs in the presence of either crotonate or butyrate and also in the absence of any added substrate.

The clearest inference from these results is that ascorbic acid plays a significant part in maintaining an intact respiratory metabolism in the liver. The efficiency of fatty acid oxidation depends upon the integrity of this respiratory metabolism. This hypothesis makes the action of ascorbic acid on fatty acid oxidation an indirect one but it serves to give a rational interpretation of the phenomena described. Further experiment alone can inform us whether ascorbic acid has a direct action on fatty acid oxidation or whether its action takes place *via* a number of intermediate steps. Such a hypothesis allows us to conclude that substances, other than ascorbic acid, and intimately associated with the respiratory metabolism of liver tissue may also affect fatty acid oxidation in a similar way; such a conclusion is obviously open to experimental verification.

Action of ascorbic acid on acetoacetic acid production by rat liver slices in presence of dl-*β-hydroxybutyric acid.*

In contrast to the effects of ascorbic acid on the oxidation of butyric and crotonic acids in presence of rat liver, there is little or no effect on the oxidation of *dl-β*-hydroxybutyric acid. Typical results are shown in Table VII. It follows

Table VII. *Effect of addition of ascorbic acid on the production of acetoacetic acid by rat liver slices in the presence of* dl-*β-hydroxybutyric acid.*

Exp.	Weight of liver taken mg. dry wt.	Thickness of slices mm.	Conc. of substrate *M*	mg. per ml. ascorbic acid added	Q_{O_2} (av. over 3 hours)	Q_{ac}
269	16·1	0·26	0·023	Nil	8·5	3·9
	16·3	0·28	0·023	0·86	10·8	4·3
270	14·4	0·27	0·023	Nil	13·4	6·3
	15·0	0·33	0·023	0·86	15·2	7·1
277	14·7	0·27	0·014	Nil	11·9	6·4
	15·8	0·29	0·014	0·86	13·8	5·8

that the oxidation of this hydroxy-acid does not depend upon the same mechanisms as those governing the oxidation of butyric and crotonic acids. This conclusion is supported by quite independent evidence, which it is hoped to report in due course in a further publication.

Action of iodoacetic acid on fatty acid oxidation by the liver.

It was found early in the course of our work on fatty acid oxidations by liver slices that iodoacetic acid (used as the sodium salt) exercised highly toxic effects at very low concentrations. Concentrations of the order of $N/5000$ will reduce the acetoacetic acid yield from butyrate or crotonate by over 50 %. The toxic action of the halogen-acid is a progressive one, the apparent inhibiting action on acetoacetic acid yield increasing with the length of exposure of the tissue to the iodoacetic acid.

On making a closer study of this phenomenon it was found that the apparent inhibitory effect of iodoacetic acid was greatly dependent on the thickness of slices used. The thicker the slice the smaller was the inhibition of acetoacetic acid production and experience showed that the most suitable thickness of slices to be used in demonstrating the highly toxic action of iodoacetic acid in an experiment of 3 hours' duration was of the order 0·2–0·3 mm. (using iodoacetic acid at a concentration about $N/5000$). Thinner slices than these suffered very rapid poisoning by the halogen acid. A series of typical results is shown in Table VIII.

Table VIII. *Action of iodoacetic acid on the production of acetoacetic acid by liver slices in presence of sodium crotonate (0·014 M).*

Exp.	Animal	Weight of liver slices taken mg. dry wt.	Thickness of slices mm.	Conc. of iodoacetic acid	Q_{O_2} (av. over 3 hours)	Q_{ac}
230	Guinea-pig	15·2	—	Nil	9·4	4·0
		15·9	—	*N*/6000	6·0	1·3
254	,,	22·7	0·33	Nil	7·7	1·8
		11·8	0·15	*N*/6000	3·4	0·3
		22·1	0·38	*N*/6000	7·8	1·4
		32·9	0·58	*N*/6000	7·7	1·8
255	,,	27·8	0·41	Nil	6·4	2·1
		31·9	0·38	*N*/7000	7·2	2·1
256	,,	17·4	0·22	Nil	7·6	2·4
		12·7	0·21	*N*/5250	5·9	1·1
257	Rat	8·6	0·18	Nil	13·9	4·3
		13·1	0·24	*N*/5250	5·7	1·6
258	Guinea-pig	14·6	0·26	Nil	7·0	2·2
		11·8	0·23	*N*/5250	3·0	1·3
260	,,	21·5	0·30	Nil	9·0	2·7
		21·3	0·27	*N*/5250	7·7	1·4

Attention may be drawn to Exps. 254 and 255 where it will be seen that with slices about 0·4 mm. thick but little change is shown in Q_{ac} in the presence of iodoacetic acid. Concentrations of iodoacetic acid higher than *N*/5000 will of course affect even the thicker slices. Exps. 256, 257, 258, 260 show that with slices of 0·2–0·3 mm. thickness, *N*/5250 iodoacetic acid brings about a drop in Q_{ac} of the order of 50 %.

Q_{O_2} is also decreased by the iodoacetic acid. A study of the rates of oxygen uptake of guinea-pig liver slices exposed to iodoacetic acid shows that the rate of oxygen uptake does not fall off immediately; the rate remains approximately constant for a variable period—45 to 90 mins.—and then falls rapidly. The phenomenon is reminiscent of that obtaining with rat liver slices (*i.e.* in the absence of iodoacetic acid). It could be explained by assuming that iodoacetic acid reacts slowly in the liver slice with some substance essential for the respiration of liver (and possibly for fatty acid oxidation) and that when the concentration of this substance falls below a certain limit there occurs a rapid fall in Q_{O_2} and Q_{ac}.

Action of ascorbic acid on guinea-pig liver poisoned by iodoacetic acid.

In view of the results already obtained with rat liver it was decided to investigate the action of ascorbic acid on normal guinea-pig liver slices exposed to iodoacetic acid.

It was found at once that the addition of ascorbic acid had precisely the same action on guinea-pig liver poisoned by iodoacetic acid as on normal rat liver. There was a prolongation of the steady rate of oxygen uptake and a decided increase in Q_{ac}. Representative results are shown in Table IX. In the presence of small concentrations of iodoacetic acid the decrease in Q_{O_2} at the end of 3 hours from that at the end of the first hour is 80 % or over. This decrease is lessened to the extent of about a half by the presence of ascorbic acid—a fact which is reflected in the increased average Q_{O_2}. The Q_{ac} may be increased as much as 200 % by the presence of ascorbic acid. We have never found however that ascorbic acid entirely neutralises the action of iodoacetic acid—the main effect, as already stated, appears to be a prolongation of the

Table IX. *Action of ascorbic acid on the production of acetoacetic acid by normal guinea-pig liver slices poisoned by iodoacetic acid.*

Substrate, sodium crotonate 0·014 *M*. The ascorbic acid was added at the same time as the iodoacetic acid to the liver slices (*i.e.* at the commencement of the experiment).

Exp.	Weight of liver slices mg. dry wt.	Thickness of slices mm.	Conc. of iodoacetic acid	mg. per ml. ascorbic acid added	Q_{ac}	Q_{O_2}	% decrease in Q_{O_2} at end of 3 hours from that at end of 1 hour
226	19·3	—	*N*/3000	Nil	0·9	4·9	80
	23·0	—	,,	0·43	2·5	8·2	40
251	13·3	0·21	*N*/7000	Nil	3·3	8·9	86
	16·7	0·22	,,	0·86	4·4	13·7	54
256	12·7	0·21	*N*/5250	Nil	1·1	6·5	80
	16·7	0·25	,,	0·86	2·2	8·5	42

maintenance of a relatively high rate of oxygen uptake. Experiments have failed so far to indicate a reaction between iodoacetic acid and ascorbic acid such as that which occurs between iodoacetic acid and glutathione.

These experiments with iodoacetic acid serve to confirm the conclusions already reached on the mechanism of action of ascorbic acid. If ascorbic acid were directly concerned with fatty acid oxidation and the toxic effect of iodoacetic acid were due to a reaction with ascorbic acid, then it would be possible to neutralise the action of the halogen acid by placing it in contact with the ascorbic acid. Such a neutralisation occurred with glutathione and iodoacetic acid in the case of oxidation of glucose by brain tissue [Quastel and Wheatley, 1932]. Ascorbic acid, however, does not neutralise the action of iodoacetic acid—even when present in considerable excess—it simply diminishes the rapidity of action of the latter.

One inference would be that iodoacetic acid in the liver slice removes a substance from the respiratory system, the production of which is greatly influenced by the presence of ascorbic acid. The substance in question would not only be essential for the maintenance of the normal respiratory level but also for the efficiency of fatty acid oxidation in the liver.

Action of glycogen on fatty acid oxidations.

In our previous paper [1933] we reported an inhibitory action of glycogen on acetoacetic acid production by guinea-pig liver slices. Over 12 experiments were carried out with consistent results. Repetition of this work 9 months later both with the same sample of glycogen and with a new sample of glycogen has failed to confirm this inhibition and at present we are at a loss to understand this irregularity of results. Our former conclusion, however, that added carbohydrate does not increase fatty acid oxidation in liver slices is not affected. Analysis of many of the livers used in the work described in this paper has not shown any distinct correlation between Q_{ac} and the glycogen content of the livers.

Summary.

1. Ascorbic acid, which is fully oxidised at 37° at p_H 7·4 in presence of oxygen, is only partially oxidised under the same conditions in presence of liver slices.

2. The presence of ascorbic acid secures an increase in Q_{O_2} and $Q_{\text{acetoacetic acid}}$ (in the latter case ranging from 30 to 200 %) of rat liver slices, when these are in a glycerophosphate-Locke medium containing sodium butyrate or crotonate.

3. The presence of ascorbic acid secures a significant increase in $Q_{\text{acetoacetic acid}}$ of scorbutic guinea-pig liver slices in media containing butyrate or crotonate; with normal guinea-pig liver slices, the increase in $Q_{\text{acetoacetic acid}}$ is small and irregular.

4. Ascorbic acid brings about a prolongation of the maintenance of the steady rate of oxygen uptake by guinea-pig and rat liver slices.

5. It is suggested that the effects of ascorbic acid on $Q_{\text{acetoacetic acid}}$ are due to the oxidation of fatty acids (*e.g.* butyric and also crotonic acids) being inherently connected with the general respiratory metabolism of the liver cell, and that ascorbic acid is important in maintaining this metabolism at a normal level.

6. Ascorbic acid appears not to affect the oxidation of *dl*-β-hydroxybutyric acid in presence of rat liver slices.

7. Iodoacetic acid at concentrations of the order of $N/5000$ has a large inhibitory action on the production of acetoacetic acid from butyric or crotonic acid by liver slices. This inhibition is reduced by the presence of added ascorbic acid, the reduction not being due to a combination between iodoacetic acid and ascorbic acid.

8. The action of glycogen on acetoacetic acid production by liver slices is discussed.

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CXLIII. THE POLYURONIDE CONSTITUENTS OF FORAGE GRASSES.

By HAROLD WILLIAM BUSTON.

From the Biochemical Department, Imperial College of Science and Technology, South Kensington.

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During the past few years, an extended study has been made in these laboratories of the chemistry of certain constituents of the forage grasses, the nitrogenous substances and the fats and waxes having received particular attention. Samples of a number of grasses of pure strain and known history being available, the opportunity was taken to examine the cell-wall substances of certain of these. Detailed information on the chemistry of these substances is extremely scanty, being limited to such rather empirical data as "crude fibre," "pentosan" and cellulose content of the tissues; in view of the importance of the cell-wall materials from the standpoint of nutritional value and digestibility of the crops, a much more detailed study seemed to be called for. The polyuronides (pectins and hemicelluloses) of cocksfoot grass have been examined in some detail, while a number of analyses of the chief cell-wall and other carbohydrate constituents of other grasses, grown under comparable conditions, are recorded.

Experimental.

Material used.

The cocksfoot used in the large-scale preparations of pectin and hemicellulose was obtained in the first place from the Agricultural Research Station of Imperial Chemical Industries Ltd., at Jealott's Hill. The crop was taken 3 weeks after the plot had been closely cut and dressed with nitrogenous fertiliser; the grass at this time was from 8 to 10 in. high, and the upper 6 in. of the blades were removed with a scythe. The first sample (1930) was minced, pressed and the residue extracted with ether and boiling alcohol, as described by Pollard *et al.* [1931]; this material was used only for preliminary experiments. The later batches (1931) were oven-dried, ground and extracted with ether only, so that the whole of the pectin, *etc.* remained in the tissue.

The material of the 1933 series was grown at the Imperial College Biological Field Station, Slough; it was allowed to grow to about 1 ft., cut low with a scythe and dried rapidly in a large steam-oven; the material, after grinding, was used without ether extraction. The small samples of various grasses used for the comparative analyses were also grown at Slough; their history is detailed below (p. 1034).

In all cases, in both large- and small-scale work, the ground dried grass was passed through sieves, and the portion of 60–90 mesh used for the extractions, experiments having shown that this was the most suitable size of particle.

Pectic substances.

250 g. of dried cocksfoot (1930) were extracted with 0·5 % ammonium oxalate on a boiling water-bath for 20 hours. The filtrate was precipitated with excess of alcohol, the gelatinous precipitate was redissolved in about 500 ml. of warm water and again precipitated with alcohol containing a little hydrochloric acid. After thorough washing with graded strengths of alcohol and drying, the precipitate was obtained as a nearly white powder (about 4 g.), containing 1·5 % of ash. Determination of the true pectin content of the substance by the calcium pectate method gave a calcium pectate value of 81 %. A sample of the calcium pectate prepared from the material gave furfuraldehyde 19·50 %, uronic anhydride ($CO_2 \times 4$) 67·10 % (average), agreeing with the values accepted for pure calcium pectate. The grass pectin was therefore considered to be of normal type.

Estimations of the amounts of the individual pectic substances present in certain of the samples were made, following the methods outlined by Branfoot [1929]. It was found necessary to vary the procedure of Branfoot in one respect. Her method includes extraction of the pectin present in the cell-wall as protopectin by means of boiling *N*/75 hydrochloric acid and estimation of the pectin in this solution as calcium pectate directly, *i.e.* without the preliminary precipitation with acidified alcohol. Unless this precipitation was carried out, the values found for "protopectin" were considerably higher than those for "total pectin" (ammonium oxalate extract), *i.e.* the *N*/75 hydrochloric acid removed from the tissue some substance capable of giving an insoluble calcium salt under the conditions of the usual calcium pectate precipitation (see below, p. 1030). It may be pointed out that this difficulty would not have arisen with the alternative methods of extraction proposed by Nanji and Norman [1928].

The results of some of these estimations are given in Table I.

Table I. *Distribution of pectic substances.*

Results expressed as g. of calcium pectate per 100 g. dry grass; mean of 3 or more values; see also Table V.

1	2	3	4	5	6
Sample no.	Total pectin (amm. oxalate extr.)	Pectin in HCl extr., (after alc. pptn.)	Pectin of middle lamella *etc.* (2–3)	"Pectin" in HCl extr., (omitting alc. pptn.)	Substance other than pectin in (5)
1930, Q	2·80	—	—	3·56	0·76
1931, 3	1·74	0·31	1·43	3·04	2·73
1931, 4	1·76	0·28	1·48	1·03	0·75

Methoxyl determinations were made on samples of pectin prepared from certain of the ammonium oxalate extracts by precipitation with acid alcohol and purified by reprecipitation, *etc.* The methoxyl values obtained were all below 3 %, after allowing for the non-pectic substances present as impurities in the material, on the basis of the calcium pectate values of the solids [*cf.* Norman, 1928]. It may be seen, however, from Table I, that by far the greater part of the pectin was extracted only by ammonium oxalate, *i.e.* occurred in the tissue as "middle lamella pectin" [Branfoot, 1929], which is usually regarded as being in the de-esterified or pectate form.

In order to obtain some further idea as to the degree of esterification of the pectin present otherwise than in the form of pectate, the following method was adopted. Ammonium oxalate extracts of the dried grass were made, the period

of extraction usually not exceeding 4 hours, at 98°; the extracts were precipitated with neutral alcohol, so that the insoluble pectic acid was not formed. The precipitate (containing ammonium pectate and soluble pectin) was well washed with 70 % alcohol and redissolved in water. In an aliquot portion of the solution, total pectin was determined in the usual manner. A further aliquot portion was treated directly with 0·4 *N* acetic acid and calcium chloride, without previous de-esterification with alkali; thus only the pectin present in the extract in the form of soluble pectate was precipitated as the calcium salt. In this manner it was possible to obtain an approximate estimate of the relative proportions of esterified and non-esterified pectin in the ammonium oxalate extracts; the assumption is made that no esterified pectin is precipitated by calcium chloride in the presence of acetic acid.

The results of these determinations, summarised in Table II, indicated that the major part of the pectic substances was indeed present in the de-esterified form. A recalculation of the methoxyl values of the esterified fractions of the various pectins, assuming that methoxyl groups were entirely absent from the "pectate-pectin" fractions, showed that (except in a single case) the average methoxyl content of the esterified pectin was still far below that required for the fully methylated derivative (11·76 % OMe). In three of the cases recorded, the calculated value was close to that required by the dimethoxyl ester, but this probably represented only a chance average figure. The unusually low methoxyl value found for sample 1930, Q, was probably due to the fact that in this case the ammonium oxalate extraction was allowed to continue for 20 hours; Norris and Schryver [1925] have shown that ammonium oxalate may produce considerable de-esterification of pectin during such prolonged periods of extraction.

Table II. *Methoxyl content of pectins.*

1	2	3	4	5
Sample	Ester-pectin as % of total pectin	Pectate-pectin as % of total pectin	Average OMe content of total pectin	OMe content of ester-pectin, %
1930, Q	40	60	0·6	1·5
1931, 3	34·5	65·5	1·93	5·59
1931, 4	21	79	1·18	5·62
1932	30	70	1·90	6·33
1933, G	24	76	2·80	11·50

It has been pointed out above (Table I) that precipitation of the extract given by *N*/75 HCl, without preliminary precipitation by acid alcohol, gave very high values for "calcium pectate," suggesting the presence of some substance giving an insoluble calcium salt under the conditions of the calcium pectate precipitation. A portion of the grass was allowed to stand at room temperature with *N*/75 HCl for 4 weeks, the acid being changed five times during the period; the acid extracts gave no trace of an insoluble calcium salt when tested. It seemed therefore that the original contaminant was not an acid liberated from an insoluble salt by the action of the *N*/75 HCl (*e.g.* oxalic acid from calcium oxalate), but that mild hydrolysis was definitely necessary for its production. Apart from this observation, the nature of the substance has not been investigated. The need, in dealing with leaf extracts at any rate, of performing a preliminary alcohol precipitation on the *N*/75 HCl extracts, as well as on those of ammonium oxalate, was evident from these results; it is not indicated by Branfoot [1929].

Hemicelluloses.

In comparison with the chemistry of the pectins, that of the hemicelluloses remains obscure; they comprise a group of substances without any very distinctive common property, and the most usual definition classes them simply as carbohydrate substances, not removed from plant tissues by water, but extracted by 4 or 5 % alkali. Many of the isolated hemicelluloses themselves are however water-soluble.

For the preparation of fairly large amounts of the hemicelluloses, the procedure of Norris and Preece [1930] was followed, and the fractions obtained have been designated according to their nomenclature. Batches of 250 or 500 g. of the dried cocksfoot were extracted first with cold water for 24 hours, then with 0·5 % ammonium oxalate for 6 hours at 98°. After washing with hot water, the well pressed residues were allowed to stand overnight with cold 50 % alcohol, containing 1 % NaOH, allowance being made for water retained in the residues [*cf.* Preece, 1931]. The alcoholic liquor was pressed out and the residue allowed to stand for several days with successive portions of 4 % sodium hydroxide solution at room temperature. The combined extracts were acidified with acetic acid, whereby the crude hemicellulose A was precipitated as a thick green flocculum, whose colloidal nature rendered its removal a matter of extreme difficulty, even in the presence of considerable excess of acetic acid. The most satisfactory separation was obtained by filtering several times on paper-pulp, on the surface of which was spread a thin layer of animal charcoal; a clear filtrate was thus obtained, while the hemicellulose A was recovered by extracting the pulp with alkali and reprecipitating with acetic acid. Considerable loss was however unavoidable at this stage. The hemicellulose was dried by means of alcohol, whereby much colouring matter was removed; the final product was still brown in colour and was found to contain 3 % of ash and 2·5 % N (Kjeldahl), equivalent to approximately 15 % of protein. Further purification was carried out by conversion into the copper compound, according to the method of Baker and Pope [1900], and products were obtained with 0·18 to 0·35 % ash and from 0·4 to 0·5 % N; further purification by this method seemed impossible.

After removal of hemicellulose A, the clear filtrates were treated with half their volume of acetone, to precipitate fraction B, and finally with excess of acetone, to precipitate fraction C; the crude fractions were in most cases converted directly into their copper compounds, and thus further subdivided into fractions B 1, B 2 and C 1, C 2 [Norris and Preece, 1930]. These substances, when dry, were obtained as cream-coloured or white powders, possessing the usual characteristics of their class; with the exception of B 1, they were readily soluble in cold water, giving light yellow solutions. B 1 was soluble only in dilute alkali but was not then reprecipitated on neutralisation. The fractions were free from N but still contained amounts of ash up to 1 %. The total yield of isolated hemicelluloses amounted only to about 60 % of the amount present in the grass, as estimated on a small sample (see Table V). From 1100 g. of dried grass the yields of the individual fractions were: A, 33 g.; B 1, 18·8 g.; B 2, 4·5 g.; B (unfractionated), 17 g.; C 1, 8·7 g.; C 2, 13·2 g.; C (unfractionated), 9·5 g.; total, 104·7 g. The yield was expected to amount to about 200 g.; the greatest losses occurred (*a*) in the isolation of fraction A and (*b*) during the purification by means of the copper compounds.

Estimations of furfuraldehyde yield and carbon dioxide yield (on distillation with 12·5 % HCl) were made by the ordinary methods on each fraction.

Measurements of rotation were also made, in the case of fractions A and B 1, in 2 % sodium hydroxide solution, in the other cases in aqueous solution. Only in the case of one sample of hemicellulose A was it possible to determine the rotation, on account of the opacity of the alkaline solutions. The results, given in Table III, are the mean of two or more determinations in each case and are calculated on an ash-free basis.

Table III. *Hemicelluloses of cocksfoot.*

1	2	3	4	5
Fraction	Grass sample	Furfuraldehyde yield %	Uronic anhydride % ($CO_2 \times 4$)	Rotation, $[\alpha]_D$
A	1931, 2	58·4	1·6	−102° (c = 1·5 %, in 2 % NaOH)
B 1	1931, 3	53·1	10·6	−47°
	1933, G	52·0	10·8	−51° (c = 1·0 %, in 2 % NaOH)
B 2	1931, 3	16·4	3·4	−53°
	1933, G	—	2·8	−12° (c = 1·4 %)
C 1	1931, 3	54·0	6·5	−83°
	1933, G	53·4	7·0	−79° (c = 1·4 %)
C 2	1931, 3	45·4	10·0	−66°
	1931, 4	—	—	−69°
	1933, G	43·6	9·8	−68° (c = 1·2 %)

In order to identify the sugar constituents of the hemicelluloses, portions of each were hydrolysed by boiling for 4 hours with 3·5 % sulphuric acid; excess of acid was removed by addition of the calculated amount of barium hydroxide solution. The filtrate in each case (distinctly acid except in the case of fraction A) was evaporated to small bulk, exactly neutralised with barium hydroxide and further evaporated to a syrup at low temperature; the syrup was extracted with warm 90 % alcohol. The alcoholic extract was evaporated again, the residue taken up in water, any barium present removed exactly and the liquid allowed to evaporate slowly *in vacuo*. In all cases except B 2, crystals readily formed in the syrups; these were removed and examined separately. The syrups were also examined for hexoses and pentoses by the usual methods. The qualitative examination of the five fractions for sugar constituents is summarised in Table IV.

Table IV. *Constituents of cocksfoot hemicelluloses.*

Fraction	Sugars, *etc.* present	Identified by	
A	Xylose	Rotation, $[\alpha]_D$ +19°. Cd xylonobromide. Osazone	Xylose crystals readily
	Arabinose	Diphenylhydrazone, M.P. 201°	
B 1	Xylose	As above	Crystals pure xylose
	Arabinose	,,	
	Galacturonic acid	Mucic acid, by HNO_3, M.P. 218°	
B 2	Galactose	Mucic acid, methylphenylhydrazone, M.P. 189°. Osazone (form of)	No crystals in syrup
	Arabinose	Diphenylhydrazone, M.P. 200°	
C 1	Xylose	As B 1, above	Xylose crystals readily
	Arabinose	,,	
	Uronic acid (?)		
C 2	Galactose	Methylphenylhydrazone, M.P. 189°. Mucic acid, M.P. 218°	Xylose crystals slowly from syrup
	Arabinose	Diphenylhydrazone, M.P. 201°	
	Xylose	Rotation, $[\alpha]_D$ = +19·5°. Cd xylonobromide	
	Galacturonic acid	Mucic acid, M.P. 218° by Br oxidation	

The identification of the uronic acid constituents of the fraction was a matter of more difficulty; in no case did the uronic anhydride content exceed 11 %, and, as is well known, the free uronic acids are very readily decomposed by dilute acid during hydrolysis. In the case of fractions A and B 2, no attempt was made to identify the very small amounts of uronic acid present. In the other cases, use was made of the bromine oxidation method of Heidelberger and Goebel [1927], using both the original hemicelluloses and the barium salts obtained after hydrolysis; the nitric acid oxidation method was also used, except in the case of fraction C 2, which was found to contain galactose.

Hemicellulose A. This fraction was hydrolysed almost entirely to xylose; a trace of arabinose was detected, probably less than 2 % of the total sugars. The hydrolysis mixture, free from acid, had $[\alpha]_D = +20°$; no hexose could be detected therein. Judging from the yield of furfuraldehyde given by the hemicellulose, since there was practically no uronic anhydride present, a small amount of hexosan was present. During the hydrolysis, an amount of dark-coloured, insoluble substance separated, equivalent to some 8–9 % of the hemicellulose. This contained 6 % of ash and gave 18 % of furfuraldehyde (on an ash-free basis).

Hemicellulose B 1. An estimation of the amount of arabinose in the hydrolysis mixture indicated the presence of about 15 % of this sugar; at most, only some 5 % of hexosan could have been present. A very small amount of mucic acid was obtained by oxidation with nitric acid; the small yield may have been due to the fact that the relatively large amount of sugar oxidation products prevented crystallisation; or another unidentified uronic acid may have been present.

Hemicellulose B 2. This fraction gave by far the lowest yield of furfuraldehyde; the hexosan content was calculated as 70 %. The total yield of the fraction was very small, and this, combined with the very low uronic anhydride content, made impossible any attempt to identify the acid constituent. It may be noted that, in this case only, the hemicelluloses prepared from different batches of grass showed markedly different rotations; this difference was further to be observed in the rotations of the hydrolysis mixtures. The 1931 preparation had $[\alpha]_D = +53°$, while that of the 1933 batch hydrolysed in a similar manner was $+17°$. The only sugars identified were galactose and arabinose; both of these are strongly dextrorotatory, so that the presence of some other sugar of low rotation was indicated. Mannose could not be detected. Judging from the yield of arabinosediphenylhydrazone given by the hydrolysis mixture some xylose was probably present, though none could be detected with certainty; the amount present however could not have accounted alone for the low rotations observed.

Hemicellulose C 1. The arabinose content of this fraction, estimated as in the case of fraction B 1, was found to be 15 %—approximately the same as in B 1. The only difference between the two fractions seemed to be in the slightly lower uronic anhydride content of this fraction. All attempts to identify the uronic acid in the hydrolysis mixture were unsuccessful.

Hemicellulose C 2. This fraction again contained much hexose, identified as galactose. Arabinose amounted to 24 % of the hydrolysate. These two sugars, with galacturonic acid, accounted for about 50 % of the hemicellulose, the remainder being xylose. Since galactose, arabinose and galacturonic acid are frequently found in association in polyuronides, the suggestion has been made that the pentose arises from the hexose by a series of mild oxidations, through the uronic acid; xylans, on the other hand, have been supposed to arise from

glucosans in a similar manner. With this in view, attempts were made to separate the xylan portion from this hemicellulose, since it seemed possible that it represented one item of a mixture, while the other three constituents were chemically combined. The methods attempted consisted in fractional precipitation, both from acid and alkaline solutions, but the furfuraldehyde, *etc.* yields of the fractions showed no significant variation from those of the original substance. On the other hand, the values obtained for the rotations of the fractions were all distinctly higher, values for $[\alpha]_D$ between $-83°$ and $-90°$ being recorded (aqueous solution, $c=0{\cdot}7$ %), so that some intramolecular re-arrangement may have taken place.

"*Combined hemicellulose.*" Preece [1931] distinguishes between "free" and "combined" hemicelluloses, the latter being defined as that fraction extracted only by hot alkali. The hemicelluloses described above were all obtained by cold extraction; from one batch however the "combined" hemicelluloses were extracted by boiling the residues after extraction with cold 4 % sodium hydroxide for 3 hours. It was found that the extract contained only a trace of hemicellulose A, fractions B and C being present in roughly equal amounts. This was somewhat unexpected, since in the isolated state fraction A is the least soluble of the hemicelluloses.

Distribution of carbohydrate fractions in various grasses.

Small samples of various grasses, grown under comparable conditions on the experimental plots at Slough, were used for a series of estimations of certain of the cell-wall and other constituents. The plots, after cutting back closely in spring (1933), were dressed with ammonium sulphate. When the blades were some 3 inches high, the plots were again cut back closely and then allowed to grow on for a further week without receiving any further ammonium sulphate. At the end of a week, the growths were again cut down, and this crop was that used for the analyses described below. (Further details of the treatment given to the experimental plots are given by Chibnall *et al.* [1933]). The samples were all rapidly dried at 98–100°, ground and sieved, the 60–90 mesh portion being used throughout. In every case, the material was re-dried in a steam-oven for 24 hours before sampling.

The following estimations were performed on each of the grasses: ash, water-soluble substances; "reducing substances" before and after hydrolysis; pectin; hemicelluloses; cellulose; xylan associated with cellulose.

1. *Ash.* Determined on portions of 0·3 to 0·5 g. of dried grass.

2. *Water-soluble substances.* 2 g. samples of dried grass were allowed to stand for 24 hours in the cold with 100 ml. of water; the extract was removed, and the residue extracted twice with 100 ml. water for 4 hours, on the steam-bath. The combined extracts were evaporated to 100 ml. Total dry matter was estimated by drying aliquot portions in the steam-oven for 48 hours.

3. *Reducing substances.* Portions of the above aqueous extract were used for estimations of free sugar (*plus* other simple reducing substances), calculated as glucose, by Bertrand's method. Similar estimations were made on portions of the extract, following hydrolysis with 1 % sulphuric acid for 40 minutes. Longer hydrolysis resulted in loss of reducing power, due possibly to destruction of fructose.

4. *Pectic substances.* 3 g. of dry material were extracted twice with 100 ml. of 0·5 % ammonium oxalate for 6 hours on the steam-bath; the residue was well washed with boiling water. Pectin was estimated in aliquot portions of the extract by the usual calcium pectate method, after preliminary precipitation with acid alcohol.

5. *Hemicelluloses.* No satisfactory standard method is yet available for the determination of this group of substances in plant tissues. In the present case they were estimated by a method

Table V. *Cell-wall constituents of various grasses.*

All figures are mean of duplicates and are calculated as % of dried grass.

Species	Date of sampling	Period of growth days	Ash %	Total water-soluble material %	Reducing sub-stances (as glucose) %	Reducing sub-stances after hydro-lysis %	Total pectin (as Ca pectate) %	Total hemi-cellulose (ash- and N-free) %	Hemi-cellulose A (ash- and N-free) %	Hemi-cellulose B (ash-free) %	Hemi-cellulose C (ash-free) %	C and B cellulose (ash-free) (including xylan) %	Xylan in C and B cellu-lose %
Rough-stalked meadow grass (*Poa trivialis*)	12. vi. 33	8	9·77	29·70	3·25	11·25	0·82	16·17	5·40	--	4·30	22·18	3·15
Timothy (*Phleum pratense*)	12. vi. 33	8	8·18	23·2	2·25	6·87	1·54	17·22	—	7·10	3·60	20·83	4·32
Chewing's fescue (*Festuca rubra* var. *fallax* Hack	9. vi. 33	8	8·32	23·0	1·01	4·70	0·90	20·70	2·76	13·55	4·37	21·38	3·89
Tall fescue (*Festuca elatior*)	9. vi. 33	8	8·55	25·5	1·87	5·62	1·62	19·92	2·77	11·02	5·70	22·56	1·86
Meadow fescue (*Festuca pratensis*)	9. vi. 33	8	7·93	24·9	1·25	6·25	1·60	18·47	3·95	9·16	5·41	24·07	3·68
,, ,,	23. vi. 33	14	—	25·1	—	3·80	2·10	17·16	2·40	8·90	5·52	—	—
Italian ryegrass (*Lolium italicum*)	12. vi. 33	8	10·1	24·7	2·87	9·05	1·04	17·60	5·26	—	2·90	23·95	3·59
Crested dog's tail (*Cynosurus cristatus*)	12. vi. 33	8	9·08	24·5	3·67	13·10	1·02	16·30	—	—	3·40	23·25	3·92
Cocksfoot (*Dactylis glomerata*)	18. xi. 33	8	8·45	20·8	—	2·50	1·60	17·90	7·81	5·13	5·76	23·05	—
,,	27. vi. 33*	—	8·16	21·9	3·54	5·51	1·97	18·31	—	—	—	33·20	8·50
,,	1931*	—	6·46	19·3	3·94	5·47	1·76	18·55	—	—	—	31·64	—

* Not previously cut; growth 12 in. high.

similar to that of Preece [1931], involving actual isolation and weighing of the fractions. To obtain concordant results it was found necessary rigidly to standardise the procedure; this precaution being observed however the method seemed satisfactory.

The residues from the pectin estimations above were air-dried and then allowed to stand for 24 hours in 50 % alcohol containing 1 % NaOH, in order to remove "lignosaccharide" [Preece 1931]. The residues from this extraction were treated exhaustively with 4 % NaOH in portions of 50 ml.; two extractions were made at room temperature, for 48 hour periods, and five further extractions on a boiling water-bath for 6 hour periods; finally the residues were boiled for 1 hour under reflux with the alkali. The total extracts were made up to 400 ml. and centrifuged in order to remove a certain amount of fine suspended matter present. The following estimations were performed:

(*a*) *Total hemicellulose.* 50 ml. of the extract were treated with 10 ml. of glacial acetic acid, followed after 30 minutes by 270 ml. of 95 % alcohol; the mixture was allowed to stand for 48 hours, when the solid was collected on the centrifuge and allowed to stand for 48 hours in 50 ml. of 80 % alcohol. The centrifuging and washing were repeated, and the solid was finally filtered off on a tared alundum crucible, washed with absolute alcohol and dried in an oven at 98° overnight. Care was taken, during this filtration, that the solid collected in a small compact mass on the crucible; this being done, the small "button" of solid was readily detached when dry. The weight of solid was corrected for ash and protein content ($N \times 6{\cdot}25$), these being estimated by the micro-methods.

(*b*) *Hemicellulose A.* 100 ml. of the extract were treated with 20 ml. of glacial acetic acid and allowed to stand for 3–4 days, and the precipitate was removed on the centrifuge. (In some cases, this was difficult; a fresh extraction, *etc.* was made in such cases.) The precipitate was washed with dilute acetic acid containing a little alcohol, then transferred to 50 ml. of 80 % alcohol, filtered and dried as before. The weight was again corrected for ash and protein.

(*c*) *Hemicellulose B.* After removal of hemicellulose A, the clear liquid (neglecting the washings) was treated with 70 ml. of acetone. After standing for 48 hours, the solid was removed, washed and dried as usual. A correction was applied for ash only.

(*d*) *Hemicellulose C.* The liquid remaining after removal of fractions A and B was precipitated with 400 ml. of 95 % alcohol, and the flocculum was removed after 48 hours, washed and dried as before. The weight was corrected for ash only.

6. *Cellulose* was estimated in portions of 2 g. by the Cross and Bevan method.

7. *Xylan in cellulose.* The furfuraldehyde yield of the Cross and Bevan cellulose was determined in each case and the xylan content of the cellulose calculated therefrom. (Hydrolysis of the Cross and Bevan cellulose from cocksfoot by the method of Monier-Williams had shown the presence of xylan in this product.) The figures given in Table V are calculated back to show the amount of this xylan in the original dry grass.

Discussion.

The pectic substances of grass have been shown to be normal in type; the amount in the tissue is definitely low. With this low pectin content is associated a relatively high content of hemicelluloses, this being in accordance with the usual observations. The relative amounts of the two classes of substance are of the order met with in lignified tissues such as timber and straws, rather than in leaves. It has been considered generally that a high content of hemicellulose is associated with a prolonged period of development, or a slow rate of growth of the tissues; that such is not necessarily the case seems indicated by the observation that grass blades of only 8–10 days' growth contain some 20 % of hemicellulose. On the other hand, such figures as have been obtained seem to show that a distinct increase of cellulose and xylan associated therewith occurs with increasing age.

With regard to the individual polyuronides, the pectic substances have been shown to exist mainly in the form of pectates; the average methoxyl content of

the pectins is low. Five hemicellulose fractions have been distinguished, but how far these can be regarded as definite entities is doubtful. Two at least of the fractions (B 1 and C 1) were very similar chemically, as regards the proportions of the constituent sugars. In the case of cocksfoot, the fractions isolated from the 1931 and 1933 batches had practically the same constants except in the case of the very small fraction B 2 (Table III), so that one may conclude that the method of separation is capable of distinguishing fairly definite fractions in the case of any particular plant tissue. The variation in fraction B 2 is not explained.

Amongst the individual sugars isolated, xylose accounts for by far the greater part. Arabinose is also widely distributed amongst the various fractions, but galactose is confined to two of the hemicelluloses. No other sugar was detected among the hemicellulose hydrolysis products, but it may be remarked that a water-soluble polysaccharide, yielding fructose on hydrolysis, was found.

Of the uronic acids, only galacturonic acid was detected with certainty, in fractions B 1 and C 2; both these fractions gave also arabinose, and it is assumed that this sugar and the uronic acid have a common origin. Although xylose was present in such large amounts in all the hemicelluloses, the corresponding uronic acid (glycuronic acid) could not be detected.

Summary.

1. The pectins and hemicelluloses of cocksfoot have been studied. Five types of the latter have been distinguished, yielding on hydrolysis mixtures of xylose, arabinose and galactose together with uronic acids. Xylose accounts for the greater part of the sugars in the hydrolysis mixture.

2. Comparative analyses of certain of the cell-wall constituents of a number of different grasses are recorded.

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CXLIV. THE ASCORBIC ACID CONTENT OF CERTAIN CITROUS FRUITS AND SOME MANUFACTURED CITROUS PRODUCTS.

By ALFRED LOUIS BACHARACH, PHYLLIS MARGARET COOK and ERNEST LESTER SMITH.

From the Glaxo Laboratories, 56 Osnaburgh Street, London, N.W. 1.

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The identification of vitamin C with ascorbic acid has evoked a number of papers in which titration methods for determination of the acid have been used to estimate the amount of vitamin C in natural products. Of particular interest in this respect are the papers concerned with the juice of citrous fruits [Bennett and Tarbert, 1933; Nelson and Mottern, 1933; Harris and Ray, 1933, 2; and Bessey and King, 1933], because of the general use and the consequent nutritive importance of these juices, because of the extent to which they enter into certain manufactured products, and also because the international standard preparation of vitamin C has been defined as the fresh juice of the lemon, *Citrus limonum*.

Our own attention was drawn to this problem somewhat indirectly; our results show general confirmation of the work of the investigators cited and certain other points that seem to us of special interest.

Ascorbic acid was determined by us as $C_6H_8O_6$, molecular weight 176, mainly with 2 : 6-dichlorophenolindophenol, by a method partly that of Harris and Ray [1933, 1] and partly that of Tillmans, whose method Harris and Ray modified. A standard solution of the dyestuff was run into the solution to be titrated; where this was a natural fruit juice, no addition was made to it, but solutions made by the extraction of portions of whole fruit were brought to the necessary degree of acidity with acetic acid. Originally we used a solution containing 0·1 g. of the dye per 100 ml., but later found that a 0·05 % solution, as suggested by Bessey and King [1933], gave equally good end-points with larger titres, and we therefore adopted it. 3 ml. portions of the juices were taken for the titration. The dye solution was standardised with ascorbic acid, the purity of which was itself checked by titration with standard iodine solution.

We also made use in some instances of titration with $N/100$ iodine solution. This was done for two reasons. In the first place, it was hoped that it might help to establish the presence of reducing substances other than ascorbic acid, since it is recognised that iodine is a less specific oxidising agent than 2 : 6-dichlorophenolindophenol; in the second place, we had been given, through the courtesy of Dr W. E. Baier, a number of results obtained by him and his colleagues in the laboratories of the California Fruit Growers' Exchange, where the iodine titration method had been used to follow through its various stages the large scale concentration of orange juice. Moreover, Dr Baier and his colleagues have themselves compared the two methods, and this has also been done by Bessey and King [1933]; it seemed therefore desirable to discover whether we would obtain the same correspondences or differences as other investigators.

Where iodine was used, 5 ml. of the fruit juice, or appropriate quantities of other products diluted with water, were taken for the titration; a few drops of fresh starch solution were added, and the iodine solution was run in slowly until a permanent blue colour was obtained. The end-point was fairly good, provided sufficient time was allowed for the titration.

Reproducibility of results.

We have investigated the reproducibility of results for both methods of titration. In most cases pairs of observations were made by two different observers; 33 pairs of determinations with 2 : 6-dichlorophenolindophenol gave a maximum variation between two observers of 0·02 mg. per ml. of juice (4 % of the mean value) and an average difference of 0·005 mg. per ml. (1 % of the mean value); the corresponding figures for 12 pairs of iodine determinations were 0·03 mg. (6 %) and 0·007 mg. (1·5 %). These figures apply to fresh fruit juices, but quite reasonably good agreement was also found for pairs of results obtained by either method on fruit extracts and manufactured products. It appears from these results that the 2 : 6-dichlorophenolindophenol method, which we agree with Harris and Ray in considering the most specific chemical test available for ascorbic acid, also gives slightly but appreciably more reproducible results than the iodine method.

Comparison of methods.

On comparing the two methods, it was found that orange and tangerine juices gave by the iodine method results that were, on the average, 1·5 % higher than those obtained with the dye, though in a number of instances the iodine results were the lower, the mean difference between all determinations by the two methods being, neglecting signs, 3·5 % of the mean value. Somewhat to our surprise, the values by the dye method for lemons were higher in every case than the values by the iodine method, the average difference being 2·5 % of the mean value. This is in direct contradiction to the results reported by Bessey and King [1933], who found a significant difference for oranges, the iodine results being higher, but no significant difference for lemons. As, however, comparisons were only made by us in quite a limited number of instances, we are not prepared to say more than that we think the matter requires further investigation. We are informed by Dr Baier (private communication) that he has found the results obtained on Californian Valencia oranges to be always higher by the iodine method than by the dye method.

Citrous fruit juices.

In Table I are summarised the results obtained by us on three different species of the genus *Citrus*. The origin of each group was taken, as far as possible, from the papers in which the fruits were wrapped; the fruits themselves were all purchased from reputable dealers, but it is, in our opinion, desirable that an investigation of this sort should be carried out on a much larger scale by those engaged in research at the actual fruit-growing centres, not only because the origin and conditions of growth of the fruit can then be confidently stated, but also because the use of large numbers of fruits would enable the results to receive the necessary statistical treatment.

In spite of the obvious handicaps attached to *ad hoc* investigations of the kind conducted by us, we think the results are self-consistent and lead to certain definite conclusions, which are discussed below.

Table I.

Nature of fruit and date of purchase	Source of fruit	No. of samples	mg. ascorbic acid per ml. juice: Range of values	mg. ascorbic acid per ml. juice: Mean value	ml. of juice per fruit: Range of values	ml. of juice per fruit: Mean value	mg. ascorbic acid in juice of one fruit: Range of values	mg. ascorbic acid in juice of one fruit: Mean value
Sweet orange (8. xii. 33)	Spain, Almeria	4	0·89	0·89	100–110	105	89–98	93
Sweet orange (seedless) (21. xii. 33)	Spain, Gandia	4	0·55–0·66	0·62	65–95	79	44–62	49
Sweet orange (8. xii. 33)	Palestine, Jaffa	4	0·53–0·63	0·58	110–125	116	61–72	67
Sweet orange (8. xii. 33)	Brazil	2	0·57–0·59	0·58	70	70	40–41	41
Sweet orange (9. xii. 33)	South Africa	4	0·34–0·68	0·57	90–115	107	39–74	60
Sweet orange (seedless) (8. xii. 33)	Spain, Tabernas	3	0·50–0·59	0·54	70–75	72	35–44	39
Sweet orange (8. xii. 33)	Brazil	4	0·40–0·42	0·40	80–90	85	34–36	35
Bitter orange (21. xii. 33)	Spain, Seville	6	0·36–0·56	0·43	25–35	31	10–17	14
Bitter orange (30. i. 34)	Spain, Seville	8	0·22–0·46	0·33	15–40	25	4–18	9
All oranges		**39**	**0·22–0·89**	**0·51**	**15–125**	**67**	**4–98**	**39**
Tangerine (8. xii. 33)	Algeria	2	0·73–0·78	0·75	35–45	40	27–33	30
Tangerine (8. xii. 33)	Spain	4	0·31–0·36	0·33	30–40	35	9–13	12
Tangerine (2. i. 34)	Jamaica	3	0·10–0·20	0·15	30–36	32	3–7	5
All tangerines		**9**	**0·10–0·78**	**0·37**	**30–45**	**37**	**3–33**	**13**
Lemon (8. xii. 33)	Spain, Malaga	3	0·62–0·73	0·68	45–60	50	33–37	35
Lemon (8. xii. 33)	Sicily, Messina	4	0·62–0·77	0·68	30–35	32	20–23	22
Lemon (8. xii. 33)	Italy	4	0·54–0·78	0·66	35–40	37	19–31	25
Lemon (21. xii. 33)	Cape Colony	4	0·47–0·62	0·54	30–40	35	17–21	19
All lemons		**15**	**0·47–0·73**	**0·64**	**30–60**	**38**	**17–37**	**24**

The figures in Table I show a very considerable range of variation for all three species of fruit. Although the range is greater for oranges than for lemons, we think this should be possibly attributed to the fact that we examined a greater number and more types of oranges than of lemons; Harris and Ray [1933, 2], who examined more lemons than oranges found a greater variation in the former than in the latter.

Stability of ascorbic acid in citrous fruits.

It seemed to us possible that there might be a loss of ascorbic acid from the plucked fruit, and that the difference between the figures of Harris and Ray [1933, 2] and our own might be due to this loss, especially since Bennett and Tarbert [1933] obtained, on the whole, higher values for the juice of freshly plucked fruits. We therefore carried out some keeping tests, without any special precautions against loss, the fruit being left in the open laboratory for approximately 4 weeks. The results are shown in Table II.

Table II. *Stability of ascorbic acid in whole fruit.*

	As purchased: No. of samples	As purchased: mg. ascorbic acid per ml.	After storage for 1 month: No. of samples	After storage for 1 month: mg. ascorbic acid per ml.
Almeria oranges	4	0·89	2	0·71
Jaffa oranges	4	0·58	2	0·52
S. Africa oranges	4	0·57	2	0·53
Tabernas oranges	3	0·54	1	0·51
Brazil oranges	4	0·40	2	0·27
Seville oranges	6	0·43	3	0·37
Spanish tangerine oranges	4	0·33	2	0·19
Averages for seven types of orange	29	**0·535**	14	**0·44**
Malaga lemons	3	0·68	1	0·67
Italian lemons	4	0·66	2	0·54
Cape lemons	4	0·54	2	0·57
Averages for three types of lemon	11	**0·63**	5	**0·59**

Concentrated orange juice.

By the courtesy of Dr W. E. Baier, we have received figures for the ascorbic acid content, determined by means of both 2:6-dichlorophenolindophenol and iodine, of orange juice immediately after expression and of the corresponding final concentrate, indicating a very small loss of potency. Although it was not possible for us to get a complete biological comparison to confirm this result, we submitted a sample of the concentrated juice to the Nutrition Department of the Pharmacological Laboratories of the Pharmaceutical Society for assay by their routine method. It was there compared biologically with a sample of pure ascorbic acid. The following figures have been obtained for this sample.

	Ascorbic acid mg. per g.
A. California Fruit Growers' Exchange	
By titration	2·54
By calculation	2·65
B. Glaxo Research Laboratory	
By titration (4. i. 34)	2·55
By titration (20. iii. 34)	2·47
C. Pharmaceutical Society	
By biological assay	2·65
By titration	2·28

The "calculated" figures from Dr Baier's laboratory are based upon the figures obtained for the fresh juice and the concentration of the finished product, as calculated from the weights of the fresh and concentrated juices. The titration figures shown were all obtained by the indicator method, though Dr Baier's technique differs from ours in certain minor details.

Marmalade.

Table III shows the result of examining a number of commercial brands of marmalade, most of which were obtained on the open market and the rest from the manufacturer, and one sample of home-made marmalade, prepared according to a well-established recipe.

Table III. *Ascorbic acid content of marmalade.*

	Apparent content of peel	Ascorbic acid (mg. per g.)	
		Indicator method	Iodine method
A	Very high	0·14	—
B	High	0·11	—
C	Medium	0·085	—
D	,,	0·085 (0·08)*	0·095
D (2nd sample)	,,	0·07 (0·07)*	0·105 (0·105)*
E	High	0·075	—
F	,,	0·07 (0·075)*	0·07
F (2nd sample)	,,	0·07	0·07
G	Low	0·065	—
H	,,	0·06	—
I (6 samples)	Jelly (orange)	<0·01	0·035 (0·035)* 1 sample only
J (6 samples)	Jelly (lemon)	<0·01	—
K (home-made)	Medium	<0·01	—

* Values in brackets by second observer.

The figures reveal at once some correlation between the apparent amount of peel present and the ascorbic acid content.

Attention may be directed to the negative results obtained with home-made marmalade, which had presumably been submitted to a lengthy process of boiling in an open vessel; in any case, the sample was approximately a year old when the test was carried out, and it is most improbable that the commercial samples had reached this age. On the other hand, the 12 samples I and J represented six different stages of maturation of the particular marmalades, and no trace of reducing power was found in any of the samples.

Orange marmalade in this country is usually made from bitter Seville oranges. We understand that quantities of fruit in the neighbourhood of 15–30 lbs. per 100 lbs. of marmalade are normally used; a simple calculation will show that only in the very richest Seville oranges does the juice contain sufficient ascorbic acid for the reducing power of the richer marmalades to be derivable from the juice alone. It seemed to us, therefore, desirable to make at any rate a preliminary survey of the distribution of vitamin C through the different parts of the orange.

Distribution of ascorbic acid in citrous fruits.

Five Seville oranges in all were examined, as well as single specimens of sweet orange, tangerine and lemon. Extracts from different portions of the outer yellow skin (flavedo), the inner thick white skin (albedo) and the pips and pith were titrated, and the results of these experiments are summarised in Table IV.

Table IV.

Ascorbic acid (mg. per g.).

Fruit	Flavedo	Albedo	Whole peel	Juice	Pips
Seville orange	2·2	0·72	1·9	0·46	<0·01
,,	1·8	0·77	0·86 (0·89)*	0·22	<0·01
,,	—	—	0·96	0·30	—
,,	—	—	0·96	0·32	—
,,	—	—	1·2	0·28	—
Sweet orange	1·3 (1·4)*	0·69 (0·68)*	1·01 (1·02)*	0·72	—
Tangerine	—	—	0·69	0·15	—
Lemon	2·1 (2·2)*	0·46 (0·51)*	1·0	1·0	—

Weights of portions.

Fruit	Flavedo g.	Albedo g.	Whole peel g.	Juice ml.	Pips g.
Seville orange (average)	10	31	41	25	30
Sweet orange	12	31	43	40	30
Tangerine	—	—	18	30	7
Lemon	—	—	20	30	20

Average distribution of ascorbic acid in whole orange (Seville) as % of total.

	%		%
Flavedo	40	Juice	15
Albedo	45	Pips	0

* Values in brackets by iodine titration.

Discussion.

The scope of our investigation naturally does not allow us to correlate the variation in ascorbic acid content of citrous fruit juices with many factors that might conceivably have a determining influence.

We have been informed that there is a considerable variation in the content of fruit solids not only in different fruits from the same tree, which might be

correlated with different degrees of ripeness, but in average fruits from neighbouring trees of undoubted genetic identity, grown under identical conditions, and there is no *a priori* reason for assuming that these variations will not apply to the ascorbic acid content.

Comparison of our results with those of Bennett and Tarbert showed that their averages for both oranges and lemons are definitely higher than ours. On the other hand, Harris and Ray found a considerably lower average value for lemons than we did. Table V summarises the figures reported by five groups of investigators for the ascorbic acid content of orange and lemon juices.

Table V.

	Oranges					Lemons				
	Maximum group range	Maximum group variation as % of mean	Total range	Maximum variation as % of mean	General mean	Maximum group range	Maximum group variation as % of mean	Total range	Maximum variation as % of mean	General mean
Bennett and Tarbert	—	—	0·91–1·03	15	0·97	0·43–0·88	70	0·43–0·94	75	0·68
Bessey and King	—	—	—	—	0·60	—	—	—	—	0·57
Harris and Ray	0·45–0·76	45	0·48–0·91	67	0·63	—	—	0·34–0·69*	73	0·47
Baier‡	—	—	0·38–0·53	33	0·45	—	—	—	—	—
Bacharach, Cook and Smith	0·34–0·68	67	0·22–0·89	130	0·51	0·54–0·78	38	0·47–0·78	50	0·63

* Omitting one extremely low value (0·18). ‡ Group values—all Valencias.

Possible causes of variation in the ascorbic acid content of orange juice are many; among them are climatic conditions, including seasonal and annual variations in sunshine, rainfall and humidity, variety of fruit, soil and other cultural conditions, size and maturity of individual fruits, method of ripening and so on.

We think the results obtained by us on fresh fruit juices establish fairly definitely the contention made by one of us recently [Bacharach, 1933] that there is a dangerous tendency to generalise on the basis of insufficient experimental evidence about the amount of a particular vitamin "naturally" present in a given foodstuff. We have obtained indications not only of a very considerable range of values of ascorbic acid in, for example, oranges of different variety and origin, but even of very marked variations between the amounts present in the juice of fruits taken at hazard from the same consignment. A certain amount of work on this subject has already been published, but it is clear that the field remains still largely virgin soil.

In contradistinction to the definite loss of ascorbic acid found by us in oranges that had been kept for 1 month, Bracewell and Zilva, using the biological test [1931], reported "no marked loss in the vitamin content [of Jaffa oranges] when the fruit is stored under ordinary conditions at about 15° for 2 months." It is, however, doubtful whether the amount of change taking place under the conditions mentioned by them would have been detected by biological assay, unless they had been able to use very much larger numbers of animals for the comparison and had been able to compare the juice before and after storage with some stable standard preparation.

We think our figures give evidence of deterioration even in so short a time as 1 month, and it seems to us a matter of some interest to those concerned with dietetics or with the fruit trade to see to what extent this change is normal and inevitable, for how long it continues, and what are the resultant limiting values for ascorbic acid content in different citrous fruit jucies. It will be remembered

that Harris and Ray [1933, 2] found an apparent increase of ascorbic acid on storing lemons and attributed it to desiccation. The two effects are clearly antagonistic and one might mask the other.

The literature contains various accounts of investigations into the anti-scorbutic potency of preserved (canned), concentrated, and dried orange and lemon juices, but in many cases the material examined was prepared on a relatively small scale. Priston [1926] described the examination of a concentrated Californian orange juice; his results suggested that there had been little destruction of its vitamin C content during processing. There were, however, no comparisons possible between the actual potency of the fresh juice and that of the manufactured product.

Goss [1925] tested biologically a number of products prepared commercially, including a vacuum-concentrated lemon juice, dried whole orange, desiccated orange juice and clarified concentrated orange juice; of the last named he reported that it "retained the antiscorbutic factor to a great extent although there is a probability that the longer exposure to the air at higher temperatures lowered this value." He used fresh orange juice as a standard antiscorbutic for control; this may have differed somewhat in vitamin C content from the fresh juice that was concentrated.

The results obtained on the sample of concentrated Californian orange juice, assayed at the Pharmacological Laboratories of the Pharmaceutical Society, indicate the possibility of conserving vitamin C even in large scale manufacturing processes. The excellent agreement between the biological and chemical assays again points to the presence in orange juice of negligible amounts of non-anti-scorbutic substances having a reducing action on the dyestuff and iodine. It may be noted that, from Dr Baier's figures for the ascorbic acid contents of the original and the concentrated juice, and the weight concentration effected, it can be calculated that about 95 % of the original ascorbic acid is retained in the final product.

We feel justified in stating on the basis of the various results reported in this paper, that the preservation of vitamin C in products such as marmalade should offer no serious difficulties in properly organised and adequately controlled manufacture. By appropriate selection of fruit containing the right quantity and distribution of vitamin C, with special reference to the proportion of peel and juice to be included in the finished marmalade, the manufacturer should be able to produce a marmalade of standardised vitamin C content, and of any value required, within the limits permitted by the natural content of the fruit. The matter is by no means one of purely academic interest; there must be many households where practically the sole sources of vitamin C available to a growing family are potatoes and marmalade; variations in the antiscorbutic potency of the latter might have an appreciable effect on the vitamin C intake of the growing child.

As far as we have been able to discover, only one biological estimation of the vitamin C in marmalade has been reported in the literature. Delf [1921] fed to a scorbutic monkey a sample of orange marmalade, made without any special precautions to prevent destruction, except that the peel and solid parts had been boiled first and the juice added at the end. Delf reported that the marmalade had "a definite but small antiscorbutic value," about 1/10th that attributed by her to fresh orange juice. Delf, like ourselves, not unnaturally assumed that the vitamin C in marmalade was derived entirely from the orange juice used.

A comparison of the antiscorbutic potency of a sample of marmalade and the juice of fresh bitter oranges similar to those from which the marmalade had

been made was carried out in 1928 in the laboratories of the Pharmaceutical Society of Great Britain [Coward, 1934, private communication]. The marmalade was found to have about one-fourth to one-third of the vitamin C potency of the bitter orange juice. As the juice was known to constitute only about one-sixth of the finished marmalade, it was evident that some other constituent was contributing to the vitamin C content. The peel of the orange was suspected on the evidence of Hess [1916] as to the presence of vitamin C in orange rind, though no further experiments were carried out to try to confirm this.

Our results seem to us to indicate that a very considerable part of the vitamin C in marmalade, where it is present to an appreciable extent, is derived from the peel rather than from the juice. There is a marked correlation between the amount of peel apparently present in the marmalade, as far as can be judged by inspection, and the amount of ascorbic acid determined by titration, in spite of the probability that there must be numerous other independent variables operating to determine the final resultant ascorbic acid. In one or two instances we obtained higher results by the iodine titration than by the 2:6-dichlorophenolindophenol method. This might conceivably be due to the production during manufacture of sugar degradation products susceptible of oxidation by iodine solution but not reducing the dye under the conditions of the test. Nor must the possibility be overlooked that there may be produced sugar degradation products capable of reducing both reagents; there is no method known at present for establishing this point, save an extensive series of comparative chemical and biological tests. The recent work of Euler and Martius [1933] and others shows that physiologically inactive reducing substances can be prepared from simple sugars by heating under alkaline conditions, but we have found it also possible to obtain an increase in the reducing power of malt extract by simply heating it for ½ to 2 hours at 100°; it is conceivable that traces of these substances might be produced during marmalade manufacture, even in the presence of natural fruit acids.

On the other hand, we have obtained some indication of the presence of inhibiting substances, or at any rate of technical difficulties in the estimation that result in the apparent presence of inhibiting substances. When pure ascorbic acid is added to orange or lemon juice, almost complete "recovery" is obtainable by either of the titration methods. This is not always the case if pure ascorbic acid is added to marmalade. Nevertheless, even bearing in mind the possible presence of reducing substances other than ascorbic acid, and of substances inhibiting the reduction of dyestuff and iodine, as well as the experimental errors inseparable from the titration of samples of marmalade, we are of opinion that the correlation between the apparent peel contents of marmalades and their reducing powers is too close to be other than significant.

Our results with the peel of citrous fruits might possibly have been attributed to the presence of reducing substances other than ascorbic acid, although the agreement between iodine and dye titrations is some evidence against such a view. We were therefore interested to find in the literature references to the antiscorbutic properties of orange peel. Hess and Fish [1914] report that "the juice of orange peel seems to serve the same purpose as the juice of the orange itself" in clinical experiments on infants. Hess and Unger [1918, 1] report that "a watery decoction of orange peel was able to protect against scurvy," and also [Hess and Unger, 1918, 2] that "orange peel possesses marked antiscorbutic potency, and withstands desiccation remarkably well." Willimott and Wokes [1926], however, found that an alcoholic extract of lemon flavedo was only feebly antiscorbutic; the conditions of preparation and storage of their

extract may well, however, have been such as to destroy any ascorbic acid present.

If our results lend themselves to the interpretation that there is a steadily falling concentration of vitamin C as one passes from the outside to the inside of the orange, it seems to us to open up some lines of investigation into the actual processes whereby ascorbic acid is synthesised in the plant. Is it possible that ascorbic acid is produced by some photosynthetic process involving the pigments themselves, and if so, which of the pigments in orange flavedo are responsible?

We shall content ourselves here with pointing to the unexpected similarity shown by the orange and the apple, since Bracewell *et al.* [1931] have fully demonstrated that the concentration of vitamin C in the tissue of Bramley's Seedling apples increases as the skin is approached from the core, and is more than six times as great in the peel as in the flesh near the core. The apparently very different morphology of the orange led us to assume, as we fancy has been assumed by other investigators, that the natural site of its vitamin C would be the juice, and that any present in the peel would be more or less adventitious. Our results suggest that this is very far from the truth.

The antiscorbutic activity of ascorbic acid has been generally accepted as about 16,000 units per g., but Key and Morgan [1933] in a recent paper reported only half this value and suggested that the discrepancy might be attributed to natural variations in lemon juice. The paper previously published by Bennett and Tarbert [1933] and the subsequent publication by Harris and Ray [1933, 2] lend some support to this suggestion, though Key and Morgan's value would necessitate that their lemon juice contained 1·35 mg. ascorbic acid per ml., a figure higher than has ever been reported by chemical determination, as indeed Harris and Ray themselves observe. The matter can never be finally settled since Key and Morgan carried out no chemical determinations of ascorbic acid on the lemon juice used for their comparisons. Qualitatively, however, we confirm the finding of Harris and Ray that fresh lemon juice as such, without further qualification, is obviously unfitted for use as an international standard preparation in vitamin testing.

The mean value found by us for lemon juice was 0·64 mg. per ml., which assigns to the international unit of vitamin C an ascorbic acid equivalent of 0·064 mg. This would give to ascorbic acid a value of nearly 16,000 international units per g. Since it is clear that our relatively small number of samples did not by any means cover the whole range of variation in lemon juices, no serious inaccuracy would be introduced into assays that have already been completed if the Permanent Commission for Biological Standardisation were to revise the international standard for vitamin C assay and assign to pure ascorbic acid an antiscorbutic activity of 10,000 or 20,000 international units per g.

Summary.

Values are presented for the ascorbic acid content of the juice of individual fruits of oranges from nine different sources, tangerines from three sources and lemons from four sources. Determinations were made by titration with 2:6-dichlorophenolindophenol in acid solution, and in many cases with iodine also. These two methods gave results in good agreement.

Thirty-nine oranges gave extreme values of 0·22 and 0·89 mg. ascorbic acid per ml. of juice, with a mean of 0·51 mg. per ml. Nine tangerines gave extreme values of 0·10 and 0·78 mg. per ml., with a mean of 0·37 mg. per ml. Fifteen lemons gave extreme values of 0·47 and 0·73 mg. per ml., with a mean of 0·64 mg.

per ml. Considerable variations were often found even between fruits from the same consignment.

Storage of the fruit for 1 month caused on the average nearly 20 % loss of ascorbic acid in oranges, but only 6 % in lemons.

Examination of a concentrated orange juice by both chemical and biological methods showed that negligible loss of ascorbic acid had occurred during concentration and subsequent storage.

Eleven varieties of marmalade contained 0·06 to 0·14 mg. ascorbic acid per g. except for three varieties, which gave a negative test. The richer kinds were those apparently containing most peel.

In all types of citrous fruits examined, the flavedo was richest in reducing power, the albedo next richest; the juice was poorer in ascorbic acid than the peel, while the pips contained no demonstrable amount.

In view of the variable antiscorbutic potency of lemon juice, it is suggested that the international unit of vitamin C should be redefined as the vitamin C activity of some definite weight of ascorbic acid.

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CXLV. THE CATALYSIS OF PEROXIDE OXIDATIONS BY FERROUS IONS.

By JOHN DEMPSTER STIRLING.

From the Institute of Physiology, The University, Glasgow.

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The importance of "oxygenase" systems in biological oxidations has been abundantly shown by the work of Engler and Wild [1897], Bach and Chodat [1902–1904], Manchot [1902], Gallagher [1923], Warburg [1921–1928], and Thurlow [1925]. The "oxygenase" theory may be briefly summarised as follows.

There exist in living cells certain autoxidisable substances capable of uniting with molecular oxygen and so giving rise to highly oxygenated peroxide-like compounds which act as powerful oxidising agents. If A represents such an autoxidisable substance and B a substance capable of acting as an oxygen acceptor, then the oxidation of B is represented by the following scheme:

$$A + O_2 \longrightarrow AO_2$$
$$AO_2 + 2B \longrightarrow A + 2BO.$$

A model for this reaction is to be found in the well-known induced oxidation of indigo to isatin in the presence of benzaldehyde, first described by Baeyer and Villiger [1900].

The transference of oxygen from the "peroxide" (AO_2) to the oxidisable substance B is made possible by the intervention of enzymic systems known as peroxidases. How these exert their specific influence is still a matter of conjecture, but it is thought that metals such as iron, copper and manganese may play a part. Warburg [1925], indeed, ascribes to iron an extremely important rôle in tissue oxidations and postulates a complete "oxygenase" system in which the iron activates molecular oxygen and brings about its transference to organic substances. Closely related is the *in vitro* function of iron as a catalyst in reactions between organic substances and oxygen which is already in an activated form. As such the function of iron is that of a peroxidase. This catalytic function of ferrous iron was first observed by Fenton [1898] who demonstrated the oxidation of tartaric acid to dihydroxymaleic acid by hydrogen peroxide in the presence of ferrous salts. Brode [1901] put forward the view that an unstable compound is first formed by combination of the ferrous salt and hydrogen peroxide and that this is the actual catalyst. Mummery [1913], investigating the reaction between ferrous salts and hydrogen peroxide, found that each gram-atom of ferrous iron required for oxidation to the ferric state one half-gram-molecule of hydrogen peroxide, and further, that hydrogen peroxide may be catalytically decomposed by addition to a solution of a ferrous salt. Mummery made use of Trillat's reagent (tetramethyldiaminodiphenylmethane in citric acid solution) which gives a blue colour with the lead dioxide class of superoxides but remains unchanged in the presence of true peroxides. He found that when hydrogen peroxide is added to a solution of a ferrous salt containing Trillat's

reagent a deep blue colour results. No colour is given when Trillat's reagent is added to a solution of a ferrous salt after the addition of hydrogen peroxide. The reagent is not coloured by ferrous salts, ferric salts or hydrogen peroxide alone or by ferric salts in the presence of hydrogen peroxide.

Mummery concluded from these findings that in the oxidation of ferrous salts by hydrogen peroxide there is an intermediate stage with the production of an unstable and extremely active oxidising agent. He suggested that this might be the actual oxidising agent in Fenton's reaction and assigned to it a perhydrol structure $HO.O—Fe—SO_4H$, ferrous sulphate perhydrol.

More recently Küchlin and Boëseken [1929], studying the oxidation of fructose and glycerol, have suggested that in such oxidation systems the first reaction is the formation of a binary complex of ferrous salt and the oxidisable substance. This may then be followed by an addition of hydrogen peroxide giving a ternary complex which is disrupted by the act of oxidation, liberating ferric iron and the oxidation product.

It is evident from the foregoing résumé that the rôle of iron in catalysed oxidations is far from clear. It is still uncertain whether the iron undergoes a change of valency while acting as a catalyst and how such a change of valency, if it does take place, is related to other reactions occurring between members of the system. The present study has been directed on both qualitative and quantitative lines towards the elucidation of the reactions involved in the catalysis of peroxide oxidations by means of ferrous iron. The qualitative observations are reported in Part I and the quantitative aspects in Part II.

I. Qualitative aspects.

Several systems of the form hydrogen peroxide—ferrous iron—oxidisable substance have been examined. Qualitative tests have been carried out to determine the fate of each member of the system, while any special features of each system having a bearing on the points at issue have been noted.

Ferrous iron was detected by means of the ferricyanide reaction and ferric iron by the colour given with thiocyanates. Hydrogen peroxide was detected by means of the blue ether-extractable colour given with chromic acid. Oxidisable substances have been used in the form of stock solutions of known strength. Dilutions were made from these as required. Ferrous salts have been used in the form of freshly prepared solutions of known strength in glass-distilled CO_2-free water. Hydrogen peroxide was used in the form of neutral dilutions from Merck's perhydrol. Each solution was standardised against $KMnO_4$ immediately before use.

The method of carrying out the examinations was as follows:

A measured quantity of the stock solution of the oxidisable substance was added to a known volume of the ferrous salt solution. This was followed by distilled water of such volume that after addition of the hydrogen peroxide solution (which was added last) the required concentrations of the reagents were attained.

After addition of the hydrogen peroxide the qualitative tests were carried out.

The oxidation of tartaric acid.

The following solutions were examined:

(1) 0·1 *M* tartaric acid which was 0·01 *M* with respect to ferrous salt and 0·01 *M* with respect to hydrogen peroxide.

(2) 0·1 *M* tartaric acid which was 0·01 *M* with respect to ferrous salt and 0·1 *M* with respect to hydrogen peroxide.

(3) and (4) as (1) and (2) but without tartaric acid.

Controls:	1	2	3	4
Hydrogen peroxide	−	−	−	+
Ferrous iron	+	+	−	−

In solutions 3 and 4 addition of peroxide gave rise to a strong orange-yellow colour which persisted—the thiocyanate test being strongly positive.

Addition of hydrogen peroxide to solutions 1 and 2 gave a greenish yellow colour which faded rapidly giving a water-clear solution free from ferric iron. A positive thiocyanate reaction was only given while the greenish yellow colour persisted in the solution.

It was noted that the time required for the disappearance of the colour produced by addition of hydrogen peroxide to a solution of tartaric acid containing ferrous iron became less with successive additions.

A typical example may be given.

0·1 *M* tartaric acid containing 0·01 *M* ferrous sulphate was taken.

0·01 *M* hydrogen peroxide was added and the time taken for the colour so produced to fade was noted by means of a stop-watch. A second proportion of peroxide was now added and the time again noted, the process being repeated until, in all, ten additions of peroxide had been made.

No. of additions of peroxide	1	2	3	4	5	6	7	8	9	10
Time in seconds	194	130	83	81	79	75	76	85	74	74
	198	128	81	81	78	76	74	72	73	72

It will be seen that the time required for the disappearance of the ferric iron initially formed decreases as the concentration of the tartaric acid decreases—the reduction therefore cannot be brought about by a reaction in which tartaric acid plays a part.

To a solution of tartaric acid containing ferrous iron there were added a few drops of Trillat's reagent (1 % tetramethyldiaminodiphenylmethane in 1 % citric acid). On addition of hydrogen peroxide a deep blue colour was formed. This, however, quickly faded giving a water-clear solution.

A solution of tartaric acid containing ferrous iron was divided into two portions *a* and *b*. To *a* a few drops of Trillat's reagent were added. Hydrogen peroxide was then added to both solutions. It was observed that the colours initially formed faded rapidly, the blue of *a* and the greenish yellow of *b*, and that the solutions became colourless simultaneously. No colour was given on the addition of Trillat's reagent to solution *b* after the fading of the greenish yellow colour. Addition of Trillat's reagent while the greenish yellow colour persisted gave the blue colour.

The above observations lead to the conclusion that the same substance is responsible for giving the ferric iron test, the greenish yellow colour of the solution and the reaction with Trillat's reagent. It appears to have but a temporary existence, and the rate of its disappearance does not appear to bear any relationship to the amount of tartaric acid present in the solution.

A further experiment bearing on the production of this labile substance may be reported.

To a solution of a ferrous salt an amount of hydrogen peroxide more than

sufficient to oxidise all the iron to the ferric state was added. Immediately the solution became brown in colour and effervescence took place. If allowed to stand the effervescence stopped and a brown precipitate formed. No ferrous iron could be detected in the solution.

If, however, a solution of tartaric acid were added while effervescence was taking place the deep brown colour of the solution faded immediately to greenish yellow. This latter colour persisted for some time and finally faded to leave a water-clear solution. Only ferrous iron could now be detected in the solution. The production of the labile substance mentioned above does not seem therefore to be conditional on the presence of ferrous iron in the solution.

The discussion of the facts reported in this section is postponed until the examination of other oxidation systems has been described. The reactions taking place during the oxidation of tartaric acid appear to proceed very rapidly and it was felt that it would be advantageous to examine systems requiring a longer time for the completion of the various phases of reaction.

The oxidation of d-*glucose.*

d-Glucose was shown to be oxidised to glucosone by Fenton's reaction by Morrell and Crofts [1899].

Preliminary study revealed that with *d*-glucose the reaction appeared to proceed at a much slower rate than with tartaric acid, and it has accordingly been chosen to illustrate certain points.

When hydrogen peroxide is added to a solution of *d*-glucose containing ferrous iron there is initially developed a yellowish brown colour. This, as shown by a positive thiocyanate test, is due to the presence of ferric iron. The colour persists for a long time but slowly disappears until finally a solution is obtained which is only slightly yellow in colour. This solution is now free from ferric iron but contains ferrous iron.

It was found that on adding sufficient hydrogen peroxide the presence of this reagent could be detected in solution. The peroxide was found to disappear quite rapidly from the solution, the time required depending on the amount of peroxide added. The disappearance of the ferric iron did not, however, coincide with the disappearance of the peroxide. Indeed a very much longer time was required for the solution to react negatively to thiocyanate. In one experiment a solution was made 0·1 *M* with respect to *d*-glucose, 0·01 *M* with respect to ferrous sulphate and 0·1 *M* with respect to hydrogen peroxide, the oxidising agent being added last. It was found that all the peroxide had disappeared from the solution in 12 minutes, while the ferric iron did not disappear until after 24 hours had elapsed.

Table I.

d-Glucose 0·1 *M* and $FeSO_4$ 0·01 *M* throughout.

No. of tube	1	2	3	4	5	6	7	8	9	10
Molarity hydrogen peroxide	0·01	0·02	0·03	0·04	0·05	0·06	0·07	0·08	0·09	0·10
Ferric iron	+	+	+	+	+	+	+	+	+	+
Ferrous iron	+	+	+	+	−	−	−	−	−	−
Hydrogen peroxide	−	−	−	−	+	+	+	+	+	+

In Table I are given the results of observations on a typical glucose system, the tests being carried out immediately after the addition of hydrogen peroxide.

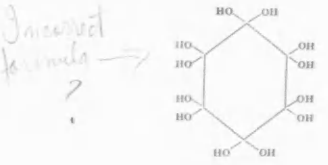
It will be seen that in every case, with additions of peroxide up to concentrations representing 4 g.-mol. proportions of hydrogen peroxide per g. atom of iron, ferric and ferrous iron were found in the solution. The intensity of the thiocyanate test increased as that of the ferricyanide test decreased. The presence of hydrogen peroxide could be detected only in those tubes from which all the ferrous iron had disappeared. Solutions 5–10 were of a uniform yellow colour; all showed the presence of hydrogen peroxide and absence of ferrous iron.

The difference in times taken for the disappearance of ferric iron and hydrogen peroxide strengthens the impression obtained in the study of tartaric acid oxidations that the reappearance of ferrous iron is not associated with the primary oxidation taking place in such systems. The amount of hydrogen peroxide required to be added before all the ferrous iron disappears should be contrasted with the 0·5 g.-mol. proportion required in the absence of an oxidisable substance. This point is dealt with more fully in Part II.

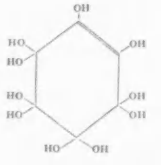
The oxidation of i*-inositol.*

So far no information has been obtained as to the behaviour of the oxidisable substance in such systems. In order to provide this, some observations may be reported on the properties of peroxide-iron systems containing *i*-inositol.

In the course of a study of the oxidation of *i*-inositol it was observed that addition of hydrogen peroxide to solutions of *i*-inositol containing ferrous iron gave rise to the production of a deep blue colour. This colour is the result of a reaction between ferric ions and the first oxidation product of *i*-inositol—an example of the general reaction between ferric ions and substances containing phenolic groups.



i-Inositol



First oxidation product
(Gives blue colour with ferric ions)

It will be readily appreciated that such systems are self-indicating so far as the presence of oxidation product is concerned.

When hydrogen peroxide is added to a solution of *i*-inositol containing ferrous iron there is initially developed a yellowish brown colour and the thiocyanate test is positive. In a short time the yellowish colour merges into green and finally into a deep blue. This colour persists for a considerable time but gradually fades leaving a water-clear solution giving no reaction for ferric iron.

In Table II is given a summary of a typical experiment with *i*-inositol.

In tubes 1–4 there was no development of blue colour, showing that no oxidation of the *i*-inositol had taken place. In these tubes the ferric iron did not disappear on standing.

In tubes 5–10 the development of the blue colour was followed by a gradual fading to a water-clear solution free from ferric iron.

Table II.

i-Inositol 0·1 *M* and $FeSO_4$ 0·01 *M* throughout.

No. of tube	1	2	3	4	5	6	7	8	9	10
Molarity hydrogen peroxide	0·01	0·02	0·03	0·04	0·05	0·06	0·07	0·08	0·09	0·10
Ferric iron	+	+	+	+	+	+	+	+	+	+
Ferrous iron	+	+	+	+	−	−	−	−	−	−
Hydrogen peroxide	−	−	−	−	+	+	+	+	+	+
Development of blue colour	−	−	−	−	+	+	+	+	+	+

There would not appear to be any oxidation of the *i*-inositol until all the iron is converted into the ferric state.

The oxidation of formic acid.

The substances examined so far, tartaric acid, *d*-glucose and *i*-inositol, yield on oxidation products which are themselves capable of further oxidation, thus introducing secondary effects. In order to rule out such secondary effects it was decided to study the behaviour of formic acid. This substance gives on oxidation carbon dioxide and water which, being incapable of further degradation, do not introduce secondary effects.

Table III summarises a series of experiments on the oxidation of formic acid.

Table III.

Formic acid 0·1 *M* and $FeSO_4$ 0·01 *M* throughout.

Tube No.	1	2	3	4	5	6	7	8	9	10
Molarity hydrogen peroxide	0·01	0·02	0·03	0·04	0·05	0·06	0·07	0·08	0·09	0·10
Ferric iron	+	+	+	+	+	+	+	+	+	+
Ferrous iron	+	+	+	+	−	−	−	−	−	−
Hydrogen peroxide	−	−	−	−	+	+	+	+	+	+

This system corresponds exactly with those already reported with the exception that although the hydrogen peroxide disappears rapidly there is no regeneration of ferrous iron. Instead, on standing, the solutions deposit a brownish yellow precipitate which is soluble in mineral acids and gives a positive test for ferric iron.

II. Quantitative aspects.

Mummery [1913] has found by means of qualitative tests that in the presence of sulphuric acid, 1 g.-mol. of ferrous iron requires for its oxidation to the ferric state 0·5 g.-mol. of hydrogen peroxide.

$$2FeSO_4 + H_2O_2 + H_2SO_4 = Fe_2(SO_4)_3 + 2H_2O.$$

In the present study ferrous iron is oxidised by hydrogen peroxide and it was desirable to find the quantitative relationships existing between these two substances under the conditions employed.

Procedure. Tubes were made up, each containing a known quantity of ferrous sulphate solution. Known quantities of hydrogen peroxide in increasing amounts throughout the series were then added. The solutions were immediately acidified with sulphuric acid and titrated with potassium permanganate solution. The results of two such experiments are shown in Table IV.

It will be seen that the permanganate titre is least in tube 7 and it may be concluded that in the absence of sulphuric acid 1 g.-mol. of hydrogen peroxide

Table IV.

Tube No.	1	2	3	4	5	6	7
g.-mol. ferrous salt	0·001	0·001	0·001	0·001	0·001	0·001	0·001
g.-mol. peroxide/ferrous salt	—	0·1	0·2	0·3	0·4	0·5	0·6
ml. 0·04868 N $KMnO_4$	19·8	15·8	12·0	8·9	5·1	2·5	0·3
	19·8	15·9	12·0	8·9	5·3	2·3	0·2

oxidises 2 g.-mol. of ferrous salt to the ferric state. The somewhat higher figure obtained in the absence of sulphuric acid is due to catalytic decomposition of some hydrogen peroxide.

The results of Part I already reported indicated that secondary reactions in the system chosen for quantitative study would give rise to complications, and in fact it was found that the only oxidisable substance whose oxidation could be followed simply and quantitatively was formic acid.

The influence of the ratio formic acid/ferrous salt on the amount of iron oxidised.

In the qualitative study of the oxidation of formic acid it was found that there was a simultaneous oxidation of ferrous iron. Reference to Table III shows that in the conditions there represented before all the iron is converted into the ferric state there must be in the system an initial concentration of 5 g.-mol. of hydrogen peroxide per g.-atom of iron. This figure was by no means constant—appearing to depend on the ratio formic acid/ferrous salt, as is shown in the following experimental series.

For each ratio ten tubes were made up, each containing the same amounts of formic acid solution and ferrous sulphate solution. Distilled water was then added so that after addition of hydrogen peroxide each tube would contain the same volume of liquid. Hydrogen peroxide solution was added finally and in increasing amount from tube 1 to tube 10. The contents of the tubes were immediately acidified with sulphuric acid, and the amount of ferrous iron remaining in each was determined by permanganate titration. Figs. 1 to 5 show the results in graphical form. In Exps. 2 and 3 (Figs. 2 and 3), solutions of a duplicate series were not acidified with sulphuric acid immediately after the addition of hydrogen peroxide, but were allowed to stand closely stoppered for 17 hours, in which time a brown precipitate settled out. They were then acidified and titrated with permanganate solution.

It will be seen that ferric iron appears in the system long before all the formic acid can have been oxidised. The oxidation of iron and formic acid is therefore simultaneous, or at least the oxidation of the former is not brought about by excess peroxide after all the formic acid has been oxidised. Moreover, the greater the amount of hydrogen peroxide in the system, the greater the amount of iron oxidised. The sharp angle of the curves after meeting the abscissa is due to the presence of excess peroxide in those solutions which were titrated immediately. It will also be seen that it is possible to have all the iron oxidised to the ferric state before sufficient hydrogen peroxide has been added to bring about the complete oxidation of all the formic acid. Although the amount of hydrogen peroxide which must be added initially before all the ferrous salt is oxidised varies greatly with the ratio formic acid/ferrous salt, Fig. 4 shows that this amount is not dependent on the absolute concentrations of the members of the system. With a high ratio formic acid/ferrous salt a larger amount of hydrogen

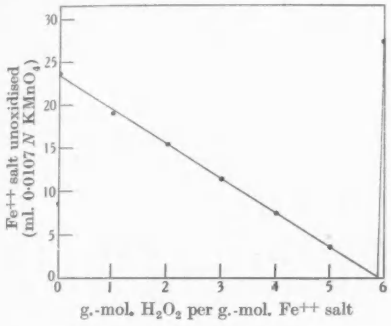


Fig 1 $\frac{\text{Formic acid}}{\text{Ferrous salt}} = \frac{20}{1}$.

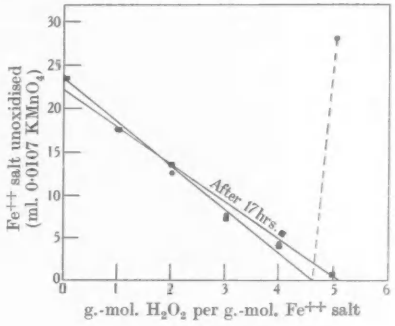


Fig. 2. $\frac{\text{Formic acid}}{\text{Ferrous salt}} = \frac{10}{1}$.

• Acidified immediately; ■ acidified after 17 hrs.

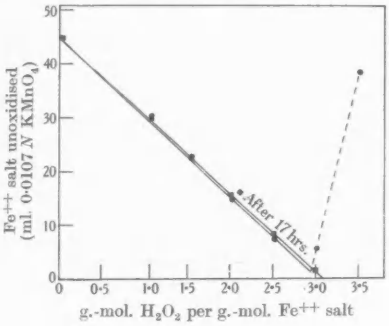


Fig. 3. $\frac{\text{Formic acid}}{\text{Ferrous salt}} = \frac{5}{1}$.

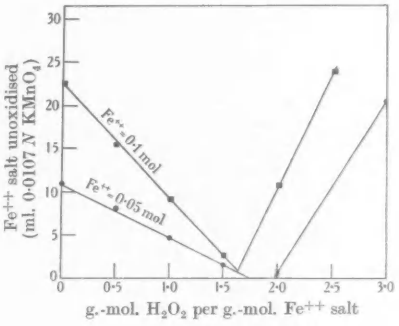


Fig. 4. $\frac{\text{Formic acid}}{\text{Ferrous salt}} = \frac{2}{1}$.

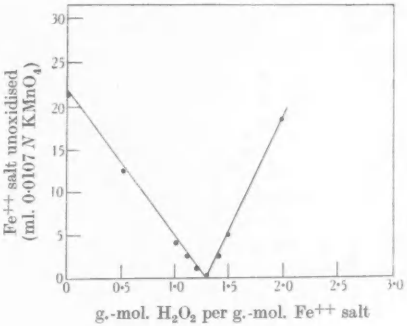


Fig. 5. $\frac{\text{Formic acid}}{\text{Ferrous salt}} = \frac{1}{1}$.

peroxide must be added to bring about oxidation of ferrous iron than with a low one. These results are roughly summarised in Table V (Fig. 6).

Table V.

Ratio formic acid/ferrous salt	20	10	5	2	1
g.-mol. H_2O_2 which must be added initially to oxidise 1 g.-atom ferrous iron	5·85	4·8	3·0	1·6	1·4

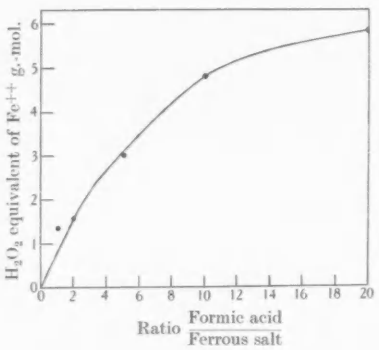


Fig. 6.

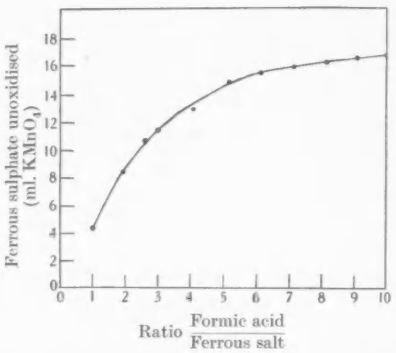


Fig. 7.

In the first series of experiments the concentrations of ferrous sulphate and formic acid were unchanged and in every case all the ferrous iron could be oxidised. The influence of the ratio formic acid/ferrous salt is also seen in the following series where the concentrations of hydrogen peroxide and ferrous salt are kept unchanged in 10 tubes while the concentration of formic acid is varied.

Table VI. (Fig. 7.)

$FeSO_4$ and H_2O_2 9×10^{-4} M throughout.

Ratio formic acid/ferrous salt	1	2	3	4	5	6	7	8	9	10
ml. permanganate solution	4·4	9·1	11·5	13·1	14·8	15·7	16	16·4	16·8	17·1

The further significance of the influence of this ratio will be considered later. Turning now to the correlation of these results with the oxidation of formic acid, the following experiments report the results of determinations of the amount of formic acid oxidised under differing conditions.

The oxidation was followed by the use of a standard baryta apparatus for the absorption of the carbon dioxide evolved. The solution containing the formic acid and ferrous salt was placed in a flask fitted with a three-holed rubber stopper carrying (1) a reflux condenser attached to a baryta absorption apparatus, (2) a burette for the introduction of hydrogen peroxide, (3) an inlet tube for CO_2-free air. The apparatus was made air-tight and free from carbon dioxide and the absorption apparatus connected. Hydrogen peroxide was introduced and the apparatus shaken for a short time. Concentrated sulphuric acid was then added, and the contents of the flask were boiled while a stream of CO_2-free air was aspirated through the solution. The amount of carbon dioxide evolved was determined by titrating the excess of baryta with standard oxalic acid solution.

It was found that (1) carbon dioxide was evolved in detectable amounts when very small quantities of hydrogen peroxide were added, (2) oxidation was inhibited by the previous addition of strong mineral acid, (3) when the ferrous salt was replaced by a normal ferric salt no oxidation took place on addition of H_2O_2, (4) if formic acid was added to a solution of a ferric salt obtained by the oxidation of a ferrous salt by the calculated amount of hydrogen peroxide, evolution of carbon dioxide followed a further addition of hydrogen peroxide.

The following table gives the results of the examination of a typical system.

Table VII.

Ratio formic acid/ferrous salt = 1. Molarity of $FeSO_4$ = 0·004.

Ratio hydrogen peroxide/H.COOH	0·5	1	1·5	2	2·5
g.-mol. hydrogen peroxide present	0·002	0·004	0·006	0·008	0·01
ml. 0·3922 *N* baryta required	5·08 5·18	9·87 10·07	15·21 15·16	18·84 18·44	20·00 19·68
g.-mol. formic acid oxidised	0·00100 0·00102	0·00197 0·00194	0·00298 0·00297	0·00369 0·00362	0·00392 0·00386
Mean	0·00101	0·00195	0·00298	0·00365	0·00389

It will be seen that the amount of formic acid oxidised is very far from being equivalent to the amount of hydrogen peroxide added. Some of the peroxide can, of course, be accounted for by the oxidation of iron which is in this case irreversible. A further series of experiments is summarised in Table VIII. In

Table VIII.

g.-mol. formic acid present	g.-mol. hydrogen peroxide present	g.-mol. ferrous salt present	ml. 0·3922 *N* baryta required to absorb CO_2		g.-mol. formic acid oxidised: Ferrous previously oxidised	g.-mol. formic acid oxidised: Ferrous not previously oxidised
0·004	0·0064	0·004	12·39	15·92	0·00243	0·00312
			12·89	16·62	0·00253	0·00326
				Mean	0·00248	0·00319
0·004	0·0084	0·004	15·02	18·76	0·00295	0·00368
			14·55	19·61	0·00285	0·00385
				Mean	0·00290	0·00376

these the oxidation of formic acid was carried out in the presence of iron previously oxidised by the requisite amount of hydrogen peroxide (0·6 g.-mol. proportion). These experiments were controlled by others in which the iron was not oxidised before the addition of formic acid.

It appears that the oxidation of formic acid by hydrogen peroxide can be catalysed by the previously oxidised iron salt. The discrepancy between the two methods was however always found. Since hydrogen peroxide disappeared from both types of system in the same way and since the amount of carbon dioxide evolved could not be increased in the first system by a longer period of aeration, the differences observed are not due to differing rates of reaction but to a smaller amount of the formic acid present actually undergoing oxidation in the first system. This is believed to be accounted for by the increased catalytic decomposition of hydrogen peroxide in the presence of the previously oxidised iron salt.

If formic acid was introduced into a solution containing initially a ferrous salt and a large excess of hydrogen peroxide, *i.e.* a solution in which the peroxide itself was undergoing catalytic decomposition, complete oxidation of the acid could be obtained provided the excess of peroxide was sufficiently large.

Table IX.

g.-mol. formic acid present	g.-mol. ferrous salt present	g.-mol. H_2O_2 present	ml. 0·3922 *N* baryta required	g.-mol. formic acid oxidised
0·004	0·004	0·04	19·56	0·00384
			19·77	0·00388
0·004	0·004	0·08	19·55	0·00393
			20·02	0·00392

In all the experiments reported the formic acid/ferrous salt ratio has been unity. It has been shown that with this ratio the amount of iron undergoing oxidation is large and that as the ratio increases, the amount becomes progressively smaller. The ratio was now varied, keeping the total volume of the solution and the amount of ferrous salt present constant and the oxidation followed as before. The results of Table X show that as the ratio increases the amount of formic acid oxidised also increases.

Table X.

Molarity of $FeSO_4$ and H_2O_2 = 0·004.

Ratio formic acid/ferrous salt	1	2	5	10	20
g.-mol. formic acid present	0·004	0·008	0·02	0·04	0·08
g.-mol. formic acid oxidised	0·00197	0·0021	0·003	0·0033	0·00343
	0·00194	0·0027	0·00293	0·00324	0·00353
Mean	0·00196	0·0024	0·00296	0·00327	0·00348
g.-mol. ferrous salt oxidised	0·0032 (*a*)	0·0023 (*b*)	0·0014 (*c*)	0·001 (*d*)	0·0008 (*e*)
g.-mol. H_2O_2 to oxidise 1 g.-atom ferrous iron	0·63	0·69	0·76	0·673	0·62

In Table XI the quantitative results obtained are correlated.

Table XI.

Ratio formic acid: ferrous salt = 1. Molarity of $FeSO_4$ = 0·004.

g.-mol. hydrogen peroxide present	0·002	0·004	0·006	0·008	0·01
g.-mol. formic acid oxidised	0·001	0·002	0·003	0·0036	0·0039
g.-mol. ferrous salt oxidised	0·00165	0·0032	0·004	0·004	0·004
g.-mol. H_2O_2 to oxidise 1 g.-atom ferrous iron	0·61	0·62	0·75	1·1	1·5

The excess hydrogen peroxide over that utilised by the formic acid is expressed in line 4, Table XI, as the amount necessary to oxidise 1 g.-atom of ferrous iron under these conditions. From a scrutiny of the table it will be seen that in this case, where the amounts of hydrogen peroxide and formic acid are molar equivalents, oxidation of the salt takes place to a greater extent than does that of the acid. The figure 0·6 g.-mol. of hydrogen peroxide for the oxidation of 1 g.-mol. of ferrous salt is obtained in Exps. 1 and 2 but not in the others. In these experiments, as the qualitative results of Table III show, there is for a short time in the solution free hydrogen peroxide. The high figures of Exps. 3, 4 and 5 can then be accounted for by catalytic decomposition of hydrogen peroxide by iron salts. The data of Table IX also support this view.

Lines 5 and 6, Table X, are compiled from the data for other values of the ratio formic acid/ferrous salt.

The figures in line 5, Table X, are calculated from the permanganate titrations reported earlier in the paper. Beside each number is given the figure from which it has been taken: (*a*) Fig. 5; (*b*) Fig. 4; (*c*) Fig. 3; (*d*) Fig. 2; (*e*) Fig. 1.

It will be seen that where all the ferrous salt has not been oxidised there is good agreement between the amount of hydrogen peroxide added to the solution and the amount equivalent to the formic acid and ferrous salt oxidised. The amount of formic acid oxidised is also seen to depend on the ratio formic acid/ferrous salt.

Discussion.

Each oxidation system which has been examined is characterised by the appearance of ferric iron. This may result in one of three ways:

(1) The change of valency of the iron may be in no way related to the catalysis.

(2) The actual catalyst may be a compound of bivalent iron, but the metal may be liberated from the catalytic reaction in the ferric form.

(3) The production of ferric iron may be a necessary preliminary to catalysis, and the catalyst may be actually a derivative of ferric iron.

The evidence is definitely in favour of the third of these possibilities.

(1) It has been shown that when tartaric acid is added to a solution of a ferrous salt containing hydrogen peroxide in amount more than sufficient for the complete oxidation of the iron, the same reaction takes place as when hydrogen peroxide is added to a solution of tartaric acid containing ferrous iron.

(2) It has been shown that when hydrogen peroxide in amount more than sufficient to oxidise all the iron is added to a solution of *i*-inositol containing ferrous iron, oxidation of the *i*-inositol takes place.

It is difficult to reconcile these two facts with the idea either that the catalyst is a ferrous iron compound liberating ferric iron after oxidation of the organic substance has taken place, or that oxidation and the appearance of ferric iron are unrelated phenomena.

It would appear that change of valency of the iron must be regarded as a necessary preliminary to the oxidation of organic substances by hydrogen peroxide when ferrous salts act in a catalytic capacity. In such systems it seems therefore that the first reaction is between the ferrous iron and hydrogen peroxide.

We have now to consider what further reactions are entered into by the ferric iron derivative, evidence for the formation of which has been reviewed above. At first sight it might seem as if we must assume the intervention of a reaction which Warburg has postulated to explain iron catalysis in the living cell, namely

$$Fe^{+++} + A \xrightarrow{\text{Oxygen}} Fe^{++} + AO$$

where A is an organic oxidisable substance.

Such a theory would require that at the end of the catalytic reaction all the iron should be in the ferrous state, for we can only conceive of the various members of the system reacting together in stoichiometrical proportions. Accordingly when the last equivalent of hydrogen peroxide in the system has raised the iron to the ferric state the reaction between the ferric compound so formed and the oxidisable substance would bring about complete reduction of the iron.

It has been observed, however, that regeneration of ferrous iron, does not take place in the case of the oxidation of formic acid, that is, with a substance

which on oxidation gives rise to products which are not further oxidisable (see Figs. 2 and 3 after 17 hours). The reduction to the ferrous state does not seem to be necessary for oxidation to take place. This conclusion is supported by further evidence. With tartaric acid systems it was shown that with subsequent additions of peroxide the rate of disappearance of ferric salt is greatly increased, apparently reaching a maximum. This cannot be explained on the basis of a reaction between ferric iron and tartaric acid, for with each subsequent addition of hydrogen peroxide the concentration of tartaric acid becomes less. If the reaction were between ferric iron and tartaric acid, according to the law of mass action, the rate of disappearance of ferric iron should decrease with each addition of hydrogen peroxide. The regeneration of ferrous iron must therefore be due to a reaction between ferric iron and a component of the system, the concentration of which increases with each addition of hydrogen peroxide. The reduction of the ferric iron must be brought about by reaction with dihydroxy-maleic acid, the oxidation product of tartaric acid.

That the reduction of the ferric iron is a secondary reaction is also shown by the fact that in the oxidation of *i*-inositol and glucose the disappearance of the ferric iron from the system is a very slow process as compared with the disappearance of hydrogen peroxide.

We are forced therefore to conclude that the ferric compound initially formed by the interaction of ferrous iron and hydrogen peroxide possesses the power of donating oxygen to oxidisable substances and that in this process there is no reversal of the initial change of valency of the iron.

Discussing now the partition of hydrogen peroxide between oxidisable substance and iron, reference to Table VI shows that as the concentration of oxidisable substance initially present in the system is increased the amount of ferrous salt finally in the system also increases. The amount of oxidisable substance undergoing oxidation correspondingly increases. It has also been shown that irrespective of the value of the ratio formic acid/ferrous salt all the iron in the system may be oxidised to the ferric state—the amount of hydrogen peroxide required varying directly with the ratio. If the theory of the catalytic function of iron presented is correct it should be possible to explain these facts in terms of it.

If the first reaction in such a system gives rise to a basic ferric salt (reaction *a*) and this salt unites with hydrogen peroxide to give an unstable iron compound which is the actual oxidising agent, then in the reaction between oxidising agent and oxidisable substance the basic ferric salt would again be produced (reaction *b*). There are then two sources of the basic ferric salt and the amount formed will depend on the amount of ferrous salt reacting with hydrogen peroxide and on the relative velocities of the reactions *a* and *b*. Any factor which would increase the rate of reaction *b* while leaving *a* unchanged would favour the production of basic ferric salt from the oxidising agent. This would then be available for further reaction with hydrogen peroxide. Such a factor would therefore tend to decrease the amount of ferrous sulphate oxidised. According to the law of mass action it is probable, therefore, that an increase in the concentration of oxidisable substance by hastening its reaction with the oxidising agent, would decrease the amount of ferrous salt undergoing oxidation.

This agrees with the known facts. It has been shown experimentally that the greater the amount of oxidisable substance in the system, the smaller the amount of ferrous salt undergoing oxidation and the greater the amount of hydrogen peroxide available for the oxidation of formic acid. It should be noted that this theory depends on a difference of velocity between the oxida-

tions of Fe^{++} salt and of formic acid which should be capable of experimental demonstration.

There is evidence that association between oxidisable substance and catalyst takes place in the systems examined. It has been noted that when hydrogen peroxide is added to solutions of ferrous sulphate there is precipitation of a brown solid which is doubtless a basic ferric sulphate produced by hydrolytic decomposition of the first product of the reaction between ferrous iron and hydrogen peroxide. This precipitation was also observed during the oxidation of formic acid. In the oxidation of tartaric acid, *d*-glucose and *i*-inositol no such precipitation was observed. Instead there was noted the reduction of the iron to the ferrous state, a process which in the case of *d*-glucose and *i*-inositol was found to be very slow. When we remember the susceptibility of the ferric derivative to hydrolytic change when no oxidisable substance is present, we can only assume that protection after the oxidation of organic substances is afforded by association with the substrate. Such association would in all probability be achieved through the free hydroxyl groups of the oxidisable substance, an assumption which might explain the precipitation observed in the oxidation of formic acid. The observations reported here as evidence of association between catalyst and substrate would be in line with the suggestion of Küchlin and Boëseken that the formation of ternary complexes is an accompaniment of the oxidation of organic substances by hydrogen peroxide in the presence of ferrous salts. It should be noted that, if such complexes are formed, the observations reported in this paper require that they should be capable of ionisation, for the thiocyanate reaction is not given by iron in a non-ionised association.

In conclusion it may be noted that the oxidation of the substrate in these systems is by no means quantitative with respect to the H_2O_2 since simultaneous oxidation of iron takes place and there is also some catalytic decomposition of hydrogen peroxide. These losses may be diminished by maintaining a high ratio substrate/catalyst.

A further source of loss is found in the reduction of iron in secondary reactions with the initial products necessitating a subsequent oxidation to the ferric state by further hydrogen peroxide. The method as used by Fenton, Morrell and Crofts, and others, depending on their own theory of the mode of the reaction, does not give the maximum amount of oxidation products. Oxidation is most favourably carried out by adding the requisite quantity of hydrogen peroxide to a solution of the substrate containing a trace of ferrous salt. When the solution gives a negative test for peroxide very little of the starting material remains unoxidised and the amount of secondary oxidation products is minimum.

Summary.

In the oxidation of organic substances by hydrogen peroxide in the presence of ferrous iron the first reaction is between the iron and the peroxide.

A derivative of ferric iron is produced and this acts as an oxygen donator.

In the reaction between this ferric derivative and the oxidisable substance there is no reduction of the iron.

The reduction of the iron is a secondary process resulting from a reaction between oxidation products and the ferric compound liberated from the reaction between oxygen donator and oxidisable substance.

There is evidence that in the catalytic oxidations considered in this paper there is formation of complexes of oxygen donator *plus* oxidisable substance.

This work was carried out by the late Dr Stirling during the tenure of the Barbour Research Scholarship in the Institute of Physiology of the University of Glasgow, and was the subject of a Thesis for the degree of Doctor of Philosophy. It was preliminary to a thorough study of the oxidation products of *i*-inositol, and it was Dr Stirling's intention to pursue this study in the quantitative manner of Part II. The quantitative studies were, however, incomplete at the time of his death on February 12th, 1933.

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CXLVI. THE OXIDATION OF THIOSULPHATE TO SULPHATE BY TISSUE SLICES *IN VITRO*.

By NORMAN WINGATE PIRIE.

From the Biochemical Laboratory, Cambridge.

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SCHMIEDEBERG [1867] isolated thiosulphate (as barium thiosulphate) from the normal urine of cats and some dogs; this observation was confirmed by Meissner [1868] and by Salkowski [1886, 2] who also found it in dog faeces. Salkowski, like most later workers, did not isolate the thiosulphate but simply showed that sulphur was formed when the urine was acidified and heated. Strümpell [1876] found thiosulphate in the urine of a patient suffering from typhoid and Spiegel [1901], who suggested that it was a normal oxidation product of cystine in the body, found it in the urine of a cystinuric. Since Mester [1890] had already shown that thiosulphate occurred in the urine after feeding both cystinuric and normal men on milk of sulphur and since several workers have found it in some human urines, the presence of thiosulphate in the urine cannot be looked upon as a characteristic of any disease.

Salkowski [1872; 1873] showed that taurine was not oxidised either to sulphate or to thiosulphate in the dog, the man or the rabbit when injected, but that it was oxidised when fed to the rabbit, and he found considerable quantities of both oxidation products in the urine. He suggested that the sulphate arose secondarily by the oxidation of the thiosulphate, for Trachtenberg [1861] had already demonstrated that the latter was readily oxidised when fed to man. Salkowski later [1876] found that isethionic acid could be oxidised to sulphate by rabbits whether fed or injected but that it only gave rise to thiosulphate when fed. These observations were extended by Heffter [1886] and Rothera [1904] who pointed out the importance of the intestinal flora in the production of thiosulphate. Heffter found that putrid meat led to a greater excretion of thiosulphate when fed to dogs than fresh meat; he also showed that diets rich in carbohydrate increased thiosulphate excretion and suggested that this was due to the increased fermentation in the gut. Some of Heffter's conclusions were harshly criticised by Salkowski [1886, 1] but his main conclusions have been generally accepted.

Inspired by Mörner's demonstration that cystine occurred in proteins Blum [1903] fed cystine to dogs and Wohlgemuth [1904, 1] fed it to rabbits; both detected thiosulphate in the urine, although Goldmann, using smaller doses, had earlier [1885] failed to do so. Blum however found great individual variations between his dogs, as might be expected if the action were due to bacteria in the gut. He also showed that no thiosulphate was excreted when the cystine was given intravenously or subcutaneously. Salkowski, returning after a long interval to his old studies, found [1914, 1] that rabbits excreted more thiosulphate in their urine when fed on cabbage than when fed on other diets; he later [1914, 2; 1917] showed that the substance concerned could be extracted from cabbage by boiling water but that if the cabbage were boiled with acid

before feeding no thiosulphate was found in the urine. He demonstrated, however, that the substance was not thiosulphate itself and that it was probably not inorganic.

It would seem therefore to be a reasonable assumption that thiosulphate is either made by bacteria in the gut or else that it is made by the body from some substance that is made in the gut.

Wohlgemuth [1904, 2] found that cystine gave rise to thiosulphate when incubated with putrid meat infusion and from this argued that it was formed during intestinal putrefaction. Neuberg and Rubin [1914] similarly found that mixed putrefactive bacteria could make thiosulphate from taurine and chondroitinsulphuric acid. A mechanism is therefore available whereby organic sulphur compounds can be converted into thiosulphate but there is little reason to think that it is the only mechanism. Ever since its discovery it has been known that hydrogen sulphide is a product of protein putrefaction. Rothera [1904] found that it was made by *Bact. coli* from cystine while Almy and James [1926] and Tarr [1933, 1] have followed its production by *Proteus vulgaris*. Tarr [1933, 2; 1934] demonstrated that other bacteria, notably *Serratia marcescens* and *C. sporogenese*, had the same power and that sulphur compounds other than cystine could be broken down. The early literature of this subject is reviewed by Wilson [1923].

Hydrogen sulphide has frequently been detected in intestinal gases and this fact, taken in conjunction with McIver, Redfield and Benedict's [1926] amplification of Teschendorf's [1924] demonstration that it is very readily absorbed from body cavities, goes to show that considerable amounts are actually formed in the intestine. This process has not been studied in detail, but Deganello [1931] has shown that there is much more hydrogen sulphide in the intestine and faeces of a rabbit that is fed on cabbage than in that of one fed on other diets. This, on the theory put forward later in this paper, is in good agreement with Salkowski's [1914, 1, 2] observations. Sulphates, and so presumably other sulphur compounds, were shown by Zörkendörfer [1931] and Kochmann [1920] to be reduced when incubated with faeces, while Wilson [1923] has shown that *Bact. typhosum* and other bacteria can reduce sulphites in the same way. Many other bacteria are known which reduce inorganic sulphur compounds to hydrogen sulphide but they have been derived from sources other than the intestinal tract. Finally Lorant and Reimann [1930], who estimated the hydrogen sulphide content of many samples of faeces, have claimed that it is possible to increase the amount of faecal sulphide-sulphur by feeding iron salts and thus, by converting some of the hydrogen sulphide into an insoluble form, minimising the absorption of it. The literature that has been cited so far shows, therefore, not only that thiosulphate may be formed in the gut but that hydrogen sulphide certainly is formed in fairly large quantities and that it is absorbed.

The early literature of the effect of administration of hydrogen sulphide to animals and men has been ably summarised by Mitchell and Davenport [1924]. The recent work that has been done on the pharmacological action of the gas is not relevant from our present point of view. Flint [1887] noticed that animals could withstand a certain critical concentration of the gas without apparent ill effect and suggested that they could oxidise it to some extent. This was confirmed and extended by Wilson [1894] who found that whereas 0·1 % of hydrogen sulphide in the atmosphere would kill a rabbit in half an hour, 0·025 % had no perceptible effect; Haggard *et al.* [1922], using anaesthetised dogs, came to similar conclusions. Haggard [1921], working *in vitro* with dog's blood, found that oxygenated blood destroyed relatively large amounts of hydrogen sulphide;

he did not determine what the end-product was but concluded that it was an acid since the CO_2-binding capacity of the blood was reduced. Becher [1927] stated that all body fluids could oxidise hydrogen sulphide to thiosulphate but he gave no experimental details, nor any data as to the rate at which this oxidation proceeded; he found that boiled blood and deproteinised blood were inactive. While Becher's conclusions are very plausible the absence of experimental details makes it difficult to assess their value. Sulphhaemoglobin, which most recent workers have found to be an artefact that does not occur *in vivo*, has been thoroughly studied by Keilin [1933]. The substance previously known as sulphhaemoglobin he looks upon as a non-dissociable complex made from oxidised haemoglobin and hydrogen sulphide or an oxidation product thereof; a simple addition compound is however made by methaemoglobin with hydrogen sulphide. It is unlikely that haemoglobin itself plays any part in the oxidation of hydrogen sulphide by blood. Denis and Reed [1927] found that sodium sulphide, when injected into an anaesthetised dog in lethal or nearly lethal doses, led to an increase in the inorganic sulphate of the serum. This increase, as might be expected, was larger when the kidneys were ligatured but was always small.

The oxidation of thiosulphate to sulphate when fed to normal men was studied by Trachtenberg [1861] and Nyiri [1923, 1]; they found that a 10 g. dose could be almost completely oxidised and that only about 5 % was excreted unchanged. Nyiri [1923, 2] used the excretion of unchanged thiosulphate after its intravenous injection as an index of kidney function, for he concluded that whereas normally 30–40 % was excreted unchanged, damaged kidneys excreted 0–23 %; he estimated the thiosulphate iodimetrically after treating the urine with animal charcoal. Holbøll [1925] criticised this method and pointed out that sometimes little or no thiosulphate was excreted by people with perfectly normal kidneys. Bacterial contamination of the urine was one cause of this but a more interesting one was increased basal metabolic rate. Holbøll gives figures for the excretion by patients with Graves' disease and other metabolic disturbances or febrile conditions. Höppener [1864] had previously found that less thiosulphate was excreted in the urine when a dose was fed during periods of fever or exercise than during periods of rest. The interpretation of the results obtained by the injection of thiosulphate is therefore difficult and the fact that, as will be shown in this paper, the oxidation can take place in both liver and kidney complicates the position still further.

A study of the processes involved in the oxidation of thiosulphate in the body is of importance, not because there is any reason to suppose that thiosulphate is a normal intermediate in the breakdown of the sulphur-containing constituents of food, such as methionine and cystine, in the body, but rather because it is an instance of the body's capacity to protect itself from poisoning by the products of intestinal putrefaction and because it is the first process of this sort that has been studied *in vitro*. The extent to which sulphur compounds are broken down to thiosulphate or hydrogen sulphide in the gut is not as yet known; but the fact that, in spite of its ready oxidisability, thiosulphate is frequently found in the urine makes it probable that this process accounts for a considerable fraction of the sulphur metabolism of the animal. This type of metabolism should be especially prominent with a very luxuriant intestinal flora, and it is significant that the rabbit has been found to excrete extra sulphate in its urine after the feeding of a much larger group of sulphur compounds than is effective in this way with the dog [Sherwin *et al.*, 1927; Pirie, 1932].

Experimental.

The technique used in the series of experiments already published [Pirie, 1934] has been altered in several respects in this series of experiments. It is unnecessary, when studying the oxidation of thiosulphate, to cut the tissue slices as thin as Warburg recommends, for there is no detectable difference in the amount of sulphate formed per mg. dry weight of tissue when the slices are cut as thinly as possible and when relatively coarse (0·5–0·7 mm.) slices are used. It is unnecessary also to use the complicated Ringer solution used by Krebs [1932], for this oxidation takes place as readily in a mixture of isotonic sodium chloride solution and isotonic sodium bicarbonate of the correct p_H. The p_H generally used was 7·6 but, as will be shown later, this need not be adhered to closely.

The method used for the estimation of sulphate is still somewhat unsatisfactory. In my previous paper the method of Cuthbertson and Tompsett [1931] was modified, and the precipitate of benzidine sulphate was washed on a filter instead of on the centrifuge. This change was made because of the difficulty experienced in making a small pellet of benzidine sulphate adhere to the end of a centrifuge-tube. This difficulty has now been overcome by the use of a faster centrifuge (3500 r.p.m.) and by washing the precipitate with 80 % acetone instead of with absolute acetone. In some recent experiments a relatively large amount of cystine and of certain tissue extractives has been precipitated along with the benzidine sulphate; this precipitate is very readily washed free from absorbed benzidine on the centrifuge but less easily on a filter. In the method of estimation used at present the technique of precipitating the benzidine sulphate and of estimating the benzidine colorimetrically is that described before, while the method of washing is that of Cuthbertson and Tompsett.

Benzidine forms a thiosulphate that is similar in many respects to its sulphate [Wahl, 1902]. This precipitates when there is more than 0·7 mg. of sodium thiosulphate in the 2 ml. sample on which sulphate is estimated. The thiosulphate is however much more soluble in 80 % acetone than benzidine sulphate and so is dissolved during the washing. The concentration of sodium thiosulphate may therefore be raised to 1 or 1·5 mg. in the 2 ml. sample without affecting the sulphate estimations. It is well known that thiosulphates are not stable in acid solution but break down to give free sulphur and a not very clearly defined group of sulphur acids; fortunately these breakdown products do not appear to precipitate with benzidine in dilute solution. Sulphate can therefore be estimated in the usual way in a solution from which the protein has been removed with trichloroacetic acid. The sulphite that is formed during the acid decomposition of thiosulphate is fairly rapidly oxidised to sulphate. The sulphate values will therefore be too high unless the estimations are started not more than half an hour after the removal of the protein. It would appear that there is no oxidation after the addition of the acetone and benzidine, for the same sulphate figures are obtained whether the estimations are left at this stage for 2 or 20 hours.

Oxidation of thiosulphate by rat tissues.

Some experiments on the oxidation of sodium thiosulphate by slices of rat tissue are collected in Table I *a*. Only liver, kidney and chorion have been found to bring about the oxidation rapidly under the conditions used, and the first is the most active. Brain, testis, spleen, blood, muscle (diaphragm) and placenta are inactive or only very feebly active. The livers used in Exps. 95 and 12

(Table I *a*) were from foetal rats about 2 and 5 days from term respectively. Clearly, foetal liver is active but less so than adult liver. It is obvious from Table I *a* that liver varies markedly in activity, and so far it has not proved possible to correlate this variation with differences in age, sex *etc.* in the rats.

The effect of inhibitors on this oxidation has not yet been studied in any detail, but the effect of a number of physical agents is recorded in the hope that at a later stage it may lead to an understanding of the mechanism of this oxidation.

Heating for 10 minutes to 60° inactivates the slices completely, and slices that have been kept at 0° for more than 2 days are inactive. These facts, taken in conjunction with the inactivity of organs other than liver and kidney, tend to confirm the theory that we have here to do with a real enzyme action and not simply with a catalytic oxidation. The enzyme system responsible for this oxidation is more stable than that concerned in the oxidation of cysteine to sulphate. Liver, for example, whether whole or sliced, will keep its activity almost unimpaired for 8–10 hours if kept cold, and the same slices which have already been used in one oxidation experiment lasting 4 hours may be used over again and show only a partial falling off in their activity. The liver used in Exp. 85 (Table I *a*) is a case in point for it had already been shaken for 4 hours at 37° with thiosulphate before Exp. 85 was put up.

A further instance of the relative stability of this system is given by its behaviour towards hypotonic solutions. Table I *b* illustrates this, for slices of the same organ are put up at the same p_H and for the same time but with the sodium chloride solution replaced by distilled water in some cases so that in these cases the tissue is suspended in a fluid with only one-sixth the normal osmotic pressure. In Exp. 75 both the oxidations were carried out under isotonic conditions but the slices used in the one marked "hypotonic" had been left with occasional shaking in distilled water for 3 hours before use. If a large volume of cold water is used and it is changed frequently the inactivation is greater. For example, the 60 mg. dry weight of slices used in Exp. 19 were washed for an hour with 250 ml. of ice-cold distilled water; after a second and a third washing with fresh cold water the activity was found to be only one-third of that of the untreated slices. Exposure to hypertonic solutions, *e.g.* 9·0 % sodium chloride solution, causes at 0° a rapid diminution in the activity of liver slices. The dry weights of tissue slices that have been shaken isotonically and hypotonically are not strictly comparable, for in the former case the wet weight is about 8·3 times the dry whereas in the latter it is about 10 times but variable. This difference in the amount of material soluble under the two conditions also shows up as a marked increase in the amount of material precipitable by trichloroacetic acid in the hypotonic fluid. Measurements of the dry weight of liver are also more or less invalid in all experiments done at a greater p_H than 8 or for periods longer than 2 hours. In spite of its apparent stability all attempts to make a preparation of this enzyme have so far been unsuccessful. Fine mincing of the liver causes a marked fall in the activity, as does freezing, while thorough grinding with sand leads to an almost complete loss of activity. It seems, therefore, that any damage to the cell structure leads to partial inactivation, and this is borne out by the fact that treatment with toluene or chloroform reduces markedly the capacity of liver to perform this oxidation. Mere subjection of the slices to high pressure on the other hand has little effect, and a sample that had been exposed to a pressure of 40 atmospheres of nitrogen for 90 minutes had Q 4·4 whereas the control Q was 5·9.

The kinetics of the oxidation of thiosulphate by rat-liver have been studied in some detail. Exps. 62 and 73 in Table I *a* show that, as might be expected,

the amount of sulphate formed is proportional to the time of action and to the amount of liver present. This regularity justifies the introduction of the symbol Q to indicate thousandths of a mg. of sulphur appearing in the form of sulphate per hour per mg. dry weight of tissue. Since Q varies with the concentration of substrate as well as with the nature of the substrate it is necessary to introduce a suffix to represent the concentration (expressed in thousandths of a mg. of sulphur per ml.) and nature of the substrate. The symbol for the conditions most commonly used in this paper, *i.e.* 3·5 mg. of hydrated sodium thiosulphate in 8 ml. of solution, is therefore $Q_{112\ \mathrm{thio}}$.

Table I *a*.

Exp. No.	Wt. of thiosulphate in 8 ml. (mg.)	Dry wt. of tissue (mg.)	Time of action min.	Wt. of sulphur oxidised to sulphate (γ)	Q
62	3·7	101 liver	30	140	2·7
	3·7	107	60	275	2·6
	3·7	83	120	420	2·5
73	4·1	10	120	55	2·7
	4·1	26	120	100	1·9
	4·1	53	120	225	2·2
85	3·5	100 (old)	120	265	1·3
90	3·5	33	64	130	3·7
95	3·5	23 foetal	120	73	1·6
12	3·5	35 liver	120	72	1·0
97	3·5	52	60	185	3·5
	3·5	73	60	225	3·1
63	3·7	82 kidney	120	245	1·5
85	3·5	40 kidney	120	200	2·5
12	3·5	20 chorion	113	78	2·1
15	3·5	17 chorion	90	64	2·5

Table I *b*.

62	3·7	Isotonic 83 liver	120	420	2·5
		Hypotonic 63	,,	400	3·2
75	3·3	Isotonic 45	120	175	2·0
		Hypotonic 76	,,	200	1·3
84	3·5	Isotonic 47	120	225	2·3
		Hypotonic 43	,,	166	1·9
85	3·5	Isotonic 40 kidney	120	200	2·5
		Hypotonic 25	,,	105	2·1
90	3·5	Isotonic 33 liver	60	130	3·7
		Hypotonic 35	,,	145	4·1
94	3·5	Isotonic 56	60	180	3·2
		Hypotonic 56	,,	120	2·1
19	3·5	Isotonic 45	60	270	6·0
	3·5	Hypotonic 60	,,	114	1·9

Table I *c*.

62	3·7	83 liver	120	420	2·5
	1·8	77	120	310	2·0
73	4·1	5·3	120	225	2·2
	2·0	75	120	200	1·3
	0·8	56	120	105	0·9
97	3·5	52	60	185	3·5
	2·7	43	60	148	3·4
	1·8	40	60	110	2·7
	0·9	42	60	65	1·5

The effect of changes in the concentration of thiosulphate is rather less simple, for the amount of sulphate formed is not quite doubled when the thiosulphate concentration is doubled. This is shown in Table I *c*. It is clear from this table that in any experiment in which much of the thiosulphate initially present has been oxidised the Q value will be smaller than it should be on account of the fall in substrate concentration. For this reason and also on account of the progressive inactivation of slices at 37° it is advisable to calculate Q values from short (1 hour) experiments.

At p_H values between 7·3 and 8 the rate of formation of sulphate is approximately constant but it falls off suddenly on either side of this range. On the acid side this diminution in activity is complicated by the spontaneous oxidation of the thiosulphate at a slow rate in even weakly acid solution. Two typical experiments on rat-liver are recorded in Table II in one of which only the

Table II.

p_H	Wt. of thiosulphate (mg.)	Dry wt. of rat-liver (mg.)	Time mins.	Wt. of sulphur oxidised to sulphate (γ)	Q
6·7	3·5	72	60	120	1·6 (1·8)
7·0	,,	69	,,	120	1·7 (2·6)
7·3	,,	65	,,	190	2·9 (2·9)
7·6	,,	73	,,	225	3·1 (3·0)
7·9	,,	62	,,	230	3·7 (3·1)
8·2	,,	(51)	,,	185	—
		Guinea-pig-kidney			
6·6	3·5	66	60	75	0·8
6·8	,,	61	,,	72	0·9
7·1	,,	95	,,	105	0·8
7·7	,,	79	,,	148	1·4
8·0	,,	57	,,	140	1·8
8·3	,,	(43)	,,	75	—

Q values are given. The p_H was controlled by varying the ratio of isotonic sodium bicarbonate to isotonic sodium chloride and, in the case of the more alkaline solutions, by using 97·5 % oxygen and 2·5 % carbon dioxide in place of the usual 95 % oxygen and 5 % carbon dioxide mixture.

In a few experiments the amount of thiosulphate disappearing was measured as well as the amount of sulphate formed. As can be seen in Table III the

Table III.

Wt. of thiosulphate in 8 ml. (mg.)	Dry wt. of rat-liver (mg.)	Time of action (mins.)	Sulphur in thiosulphate oxidised (γ)	Sulphur in sulphate formed (γ)	Q
3·5	71	240	520	430	1·5
,,	60	120	248	230	1·9
,,	56	60	146	180	3·2
,,	50*	60	180	170	3·4
,,	56†	60	130	120	2·1

* Slices cut very thinly. † Hypotonic.

agreement is reasonably good. The thiosulphate was estimated by removing the protein from a sample of the reaction mixture with zinc sulphate and sodium hydroxide as in the blood-sugar method of Hagedorn and Jensen. An excess of an acid solution of iodine in potassium iodide was then added and the excess

titrated with standard thiosulphate solution. Tissue extractives such as glutathione will of course interfere, but under the aerobic conditions used in these experiments such substances will be largely in the oxidised state, and in any case they are quantitatively insignificant. A more serious error is caused by the fact that only one-third or one-fourth of the thiosulphate is oxidised in most experiments and one is therefore measuring, rather inexactly, a small change in a fairly large quantity. It is clear from Table III that, allowing for the probable errors, there is no evidence for the existence, under these conditions, of any process leading to the destruction of appreciable amounts of thiosulphate except the formation of sulphate. This demonstration raises the question of the possible rôle of the best known oxidation product of thiosulphate, namely, sodium tetrathionate, in this oxidation. Sodium tetrathionate was prepared in the usual way by pouring a strong aqueous solution of sodium thiosulphate into an alcoholic solution of iodine. The precipitated tetrathionate was washed well with alcohol, taken up in water and precipitated again with alcohol; it had the theoretical sulphur content. As Table IV *a* shows, sodium tetrathionate is oxidised to sulphate by rat-liver and kidney but at a very much slower rate, considering the amount of sulphur that is present in 1 ml. of solution, than sodium thiosulphate, *i.e.* $Q_{143\,\text{tetra}}$ is 1·4–3·2 whereas $Q_{112\,\text{thio}}$ is 2·5–5·9. It is therefore unlikely that tetrathionate is an intermediate in the oxidation of thiosulphate to sulphate. It is possible that oxidation to sulphate is preceded by a reduction of the tetrathionate to thiosulphate. I showed in the earlier paper that such a preliminary reduction took place in the case of the oxidation of cystine but so far I have been unable to demonstrate it in this case. It is unlikely that such a reduction takes place, for the ratio $Q_{\text{thio}}/Q_{\text{tetra}}$ appears to be constant irrespective of the treatment that the slices of liver have undergone. In the case of cystine, on the other hand, it was found that the capacity of liver slices to reduce cystine to cysteine was strictly limited and that this limited the production of sulphate. The analogy is of course not wholly satisfactory, since thiosulphate, unlike cysteine, cannot undergo aerobic re-oxidation, and so it might be expected that it would be easier for the liver to maintain the concentration of the reduced substance. When incubated anaerobically with liver slices, sodium tetrathionate does not give rise to any appreciable amount of thiosulphate. Some further experiments illustrating this type of oxidation are given in Table IV *b*. All the experiments lasted for an hour and were carried out in 97·5 % O_2, 2·5 % CO_2, but the slices used in Exps. *c* and *d* are those that had already been used in *a*, while those used in *e* and *f* had already been used in *b*. Clearly the $Q_{\text{thio}}/Q_{\text{tetra}}$ ratio is approximately constant, and previous exposure to tetrathionate leads to no greater loss of activity than exposure to thiosulphate. The other thionic acids have not been tried, and sodium sulphite is so rapidly autoxidised in the absence of tissue, and still more in the presence of boiled tissue, that a study of its enzymic oxidation is not possible by this technique.

The falling off in the activity of a tissue during the second hour of shaking is especially marked in the case of livers which are initially very active. Such a fall is clearly seen in Tables III and IV *b* and is analysed more fully in Table IV *c*. There are three possible explanations of this phenomenon; firstly it may simply be the normal ageing process accelerated by the higher temperature at which the liver has been kept; secondly it may be due to the using up of a metabolite during the course of the oxidation of thiosulphate; and thirdly it may be due to the loss by diffusion of a soluble substance, either a metabolite or a co-enzyme, essential for sulphate formation. The results recorded in Table IV *c* are strongly

Table IV *a*.

Exp. No.	Wt. of tetrathionate (mg.)	Dry wt. of tissue (mg.)	Time of action (mins.)	Wt. of sulphur oxidised to sulphate (γ)	Q
84	2·4	56 liver	120	165	1·4
	2·4	45 liver heated to 70°	,,	5	—
85	2·4	42 kidney	,,	95	1·1
	2·4	28 kidney heated	,,	6	—
	2·4	No tissue	,,	0	—
90	2·7	42 liver	64	92	2·0
	5·4	40	,,	125	2·9
	1·3	41	,,	70	1·6
	2·7 (hypotonic)	43	,,	108	2·3
3	2·4	91 liver	60	160	1·8
4	2·4	37 liver	60	45	1·2

Table IV *b*.

7 *a*	3·5 thio.	53 liver	60	315	5·9
b	2·4 tetra.	57 liver	,,	185	3·2
c	3·5 thio.	24 liver	,,	73	3·0
d	2·4 tetra.	29 liver	,,	45	1·5
e	3·5 thio.	22 liver	,,	75	3·2
f	2·4 tetra.	35 liver	,,	55	1·6

Table IV *c*.

11 *a*	3·5 thio.	43 liver	60	240	5·6
b	3·5	43	120	400	4·7
c	3·5	43	60	185	4·3
d	3·5	37	,,	160	4·3
e	3·5	29	,,	115	4·0
f	3·5	45	75	120	2·1
g	3·5	28	,,	55	1·6
12 *a*	3·5	59	60	95	1·6
b	3·5	52	,,	102	2·0

in favour of the first theory. Exps. *a* and *b* were carried out in the usual way for periods of 1 and 2 hours respectively. Exp. *c* is a 1 hour experiment on the slices that had already been used in Exp. *a*. This gives $Q=4{\cdot}3$ instead of the initial 5·6. The Q values found in Exps. *d* and *e* should be compared with this value for in each case the slices underwent a preliminary shaking for one hour in thiosulphate-free bicarbonate-saline. After this, thiosulphate was added to the one (*d*) while the slices were removed from the other, rinsed and added to fresh solution containing thiosulphate. Clearly the values of Q in Exps. *c*, *d* and *e* are very similar; this shows that the presence of thiosulphate leads to no increase in the rate of disappearance of essential metabolites, for if it did Q in Exp. *c* would be smaller than in Exps. *d* or *e*. Similarly there is little loss by diffusion for if there were *e* should be smaller than *d*. Exps. *f* and *g* were carried out in the same way but with 2 hours' preliminary shaking at 37°. In Exp. *f* the oxidation was carried out in the fluid in which the slices had previously been shaken for 90 minutes, while in *g* fresh fluid was used. The difference, though definite, is small enough to indicate that this is not an important cause of loss of activity. If a gas mixture consisting of 95 % N_2 and 5 % CO_2 is used for the preliminary shaking, it is found that the slices are completely inactivated in an hour whether thiosulphate is present or not. Anaerobic incubation for half an hour causes nearly complete inactivation. Other oxidising systems are

already known which are stable only in the presence of oxygen, but this one is specially interesting since, as will be shown later, the oxidation of thiosulphate does not lead to any increase in the oxygen consumption of the slices. On account of this inactivation there is no anaerobic formation of sulphate. The presence of $M/4000$ methylene blue and $M/200$ nitrate leads neither to the production of sulphate, although the methylene blue is kept in the oxidised state by the nitrate-reducing system in the liver, nor to an increase in the stability of the enzyme system. Methylene blue and nitrate, whether singly or together, have also no effect on the aerobic sulphate production.

Manometric experiments with rat-liver.

In collaboration with Dr H. A. Krebs of this laboratory some experiments on the influence of thiosulphate on the oxygen uptake of liver have been carried out. These have led to the unexpected result that, under conditions where oxygen is appearing in the form of sulphate at a rate corresponding to one-third of the total respiration, there is no apparent increase in the oxygen consumption. Table V gives the results of one such experiment. The fluid used was Ringer

Table V.

Wt. of tissue (mg.)	Wt. of thiosulphate in 3 ml. Ringer (mg.)	Wt. of sulphur oxidised to SO_4 per hour (γ)	Q_{thio}	Oxygen equivalent of this sulphur reduced to 40 mins. (μl.)	Oxygen consumption in 40 mins. (observed) (μl.)	Q_{O_2}
7·9	1·3	27	3·4	15·7	44·7	8·5
9·7	1·3	31	3·2	18·0	56	8·7
16·1	None	—	—	—	95	8·85
7·9	None	—	—	—	45·5	8·6

solution made up according to Krebs [1932] but with magnesium sulphate replaced by magnesium chloride and with only 2 ml. of 1·2 % sodium bicarbonate per 100. Such a solution maintains a suitable p_H when used in conjunction with pure oxygen and with a side-bulb to absorb CO_2. Sulphate estimations were done on the reaction fluid in the usual way and the oxygen equivalent is calculated on the basis of 5 oxygen atoms to 2 atoms of sulphur as required by theory. This value is multiplied by 2/3 since the oxygen absorbtion was only followed for 40 minutes while the tissue was in contact with thiosulphate at 37° for an hour. The constancy in the values of Q_{O_2} in the four experiments recorded shows that no extra oxygen can have been taken up during the oxidation of the thiosulphate.

Oxidation by the tissues of other animals.

A few experiments have been made with other animals. It is unnecessary to give the results in detail and the Q values only are recorded.

	Q
Ox-kidney (2–3 hours after slaughter)	1·3
Rabbit-liver...	1·3
Rabbit-kidney	0·65
Guinea-pig-kidney	1·1–2·7
Pig-liver (2–3 hours after slaughter)	1·0
Dog-kidney (after chloroform)	0·6
Dog-liver (after chloroform)	0·8

All these experiments were done at p_H 7·6 and with 112γ sulphur present in the form of thiosulphate per ml. The effect of changes in p_H on the activity of

guinea-pig-kidney is recorded in Table II whence it is clear that the enzyme system found there is, from this point of view, similar to that found in rat-liver. The other tissues have not been studied further.

Oxidation by goose-kidney.

Goose-kidney resembles rat-liver closely in its ability to oxidise thiosulphate to sulphate. The results of a few experiments are given in Table VI; these were

Table VI.

Exp. No.	Wt. of thiosulphate (mg.)	Wt. of goose-kidney (mg.)	Time of action (mins.)	Wt. of sulphur oxidised to SO_4 (γ)	Q
78	4·0	90	120	650	3·6
79	4·0	55	120	360	3·3
	4·0	25	120	160	3·2
	2·4	68	120	280	2·1
	1·2	70	120	180	1·3
88 p_H 6·7	3·5	72	120	385	2·8
p_H 7·3	3·5	46	120	300	3·3
p_H 7·7	3·5	65	120	410	3·2
p_H 8·0	3·5	54	120	380	3·5

carried out in the usual way at p_H 7·6 but using a mixture of 1·2 % sodium bicarbonate and 1·1 % sodium chloride as the reaction fluid. As with rat organs no sulphate is made by slices that have been heated to 60° nor if the slices are shaken with thiosulphate anaerobically. Freezing and grinding cause a very marked diminution in the activity while the effect of treatment with distilled water is definite though less marked.

Goose-kidney also resembles rat-liver and kidney in its ability to oxidise cysteine and methionine to sulphate; this oxidation will be described in a later paper.

Discussion.

Although the oxidation of thiosulphate by bacteria was first observed many years ago the mechanism of the process is still obscure. Several species of bacteria are known which have this power. Lieske [1912] and Guittonneau [1925, 1, 2] have isolated such organisms from pond mud and soil but they did not study the oxidation in any detail. Nathanssohn [1902] and Trautwein [1921; 1924] have studied similar bacteria and find that the oxidation proceeds through tetrathionate and dithionate. Beijerinck [1904], on the other hand, working with *Thiobacillus thioparus*, found that tetrathionate was less readily oxidised than thiosulphate. The behaviour of this organism is therefore analogous to that of liver. A comparative study of the properties and stability of the enzyme systems derived from these bacteria and from liver would be very interesting.

Thiosulphate can scarcely be oxidised directly, for in that case 5 atoms of oxygen would have to be present simultaneously for the oxidation of one molecule of thiosulphate; furthermore, if the oxidation were direct it would be hard to see why it should cause no increase in the oxygen consumption of liver slices. The irreversible inactivation of liver slices on exposure to nitrogen is especially interesting in this connection. It is known that the respiration of cells is, in general, irreversibly inhibited by anaerobic incubation and Warburg *et al.* [1926] have found that even tumour tissue will not respire after 4 hours without oxygen or glucose. These facts suggest that the oxidation of thiosulphate

is a phenomenon which parallels closely the fundamental oxidation processes of the cell, but the connection is incomplete since some organs, with relatively high respiration rates, cannot bring about this oxidation. All attempts to reactivate liver that has been inactivated in this way have so far been unsuccessful but further work on this problem is in progress.

Summary.

Rat-liver, kidney and chorion, and goose-kidney, can oxidise sodium thiosulphate to sulphate.

This oxidation is accompanied by no perceptible increase in the oxygen consumption of the tissue slices, but it will not proceed anaerobically. The slices are irreversibly inactivated by anaerobic incubation.

Sodium tetrathionate is also oxidised, but reasons are given for thinking that it is not an intermediate.

The reason for the loss of activity by the slices on keeping and the effects of some physical agents on the enzyme system have been investigated.

The liver and kidney of some other animals have been tested and found to be active but relatively weak.

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CXLVII. THE RELATION OF THE COLOUR AND VITAMIN A CONTENT OF BUTTER TO THE NATURE OF THE RATION FED.

I. INFLUENCE OF THE RATION ON THE YELLOW COLOUR OF THE BUTTER.

By STEPHEN JOHN WATSON.
I.C.I. Agricultural Research Station, Jealotts Hill, Berks.
GERALD BISHOP and JACK CECIL DRUMMOND.
University College, London.

II. THE CAROTENOID AND VITAMIN A CONTENTS OF THE BUTTER.

By ALBERT EDWARD GILLAM
and ISIDOR MORRIS HEILBRON.
Chemistry Department, Manchester University.

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I. INFLUENCE OF THE RATION ON THE YELLOW COLOUR OF THE BUTTER.

Under the normal system of feeding dairy cows in winter in this country a most noticeable feature is the pale colour of the milk, a fact greatly emphasised in the butter made from it. The intensity of the yellow colour of the milk fat is positively correlated [Watson *et al.*, 1933] with the carotene content of the butter and also with its vitamin A content. The inclusion in the ration of some foodstuff capable of increasing the yellow colour of the milk fat is therefore of considerable importance. The oil cakes and cereals in common use are incapable of bringing about this result, for which the use of some foodstuff rich in carotene is needed. Carrots themselves may be used, but there are difficulties in the way of their adoption in general practice. In one case examined the milk of the herd produced a butter with a total yellow colour of 3·2 Lovibond units[1] which was raised to 7·2 units by feeding about 10 lb. of carrots per head daily.

In an experiment carried out in the winter of 1931–32 [Watson *et al.*, 1933] and also reported in this *Journal* [Gillam *et al.*, 1933], it was shown that artificially dried grass was a useful foodstuff in this regard when it formed 75 % of the total concentrated foodstuffs fed. This work has been extended in 1932–33 to test the value of different methods of conservation of pasture grass and their effect, in the ration, on the colour of butter.

[1] The yellow colour is stated in all cases as the Lovibond yellow units corresponding to a 1 cm. thickness of the fat. For the measurement it is diluted with three times its volume of light petroleum, the actual reading being multiplied by four.

In the first experiment four groups of Shorthorn cows were used. All groups received a basal ration of hay and roots. Group D1 acted as control and received a normal winter ration of concentrated foodstuffs, balanced for milk production, at the rate of 3½ lb. per gallon of milk produced. Groups E1, E2 and E3 received 10 %, 25 % and 50 % respectively of their concentrate ration in the form of artificially dried grass, the mixture being so adjusted that the same amounts of nutrients (starch equivalent and protein equivalent) were fed per gallon of milk to all four groups. The values obtained for the total yellow colour of the butter are given in Table I, and are shown in Fig. 1.

Table I. *Total yellow colour of the butter from groups of Shorthorn cows on a normal winter ration, and on rations containing varying amounts of artificially dried grass.*

Stated as Lovibond units.

Week ending	D 1 Control	E 1 (10 % dried grass)	E 2 (25 % dried grass)	E 3 (50 % dried grass)
17. xi. 32*	6·8	8·8	8·0	7·2
15. xii. 32	7·2	8·4	9·2	12·8
29. xii. 32	6·8	8·4	8·4	12·0
23. i. 33	6·0	7·6	7·6	10·0
13. ii. 33	4·4	6·0	6·0	9·2
2. iii. 33	4·8	6·4	5·2	8·4
27. iii. 33	5·2	6·0	5·2	9·6
Averages	5·7	7·1	6·9	10·3

* All four groups on control ration.

The total yellow colour of the butter has not been markedly affected by the inclusion of 10 and 25 % of artificially dried grass in the concentrate ration, but the group receiving 50 % of its concentrates in the form of this material shows a much greater improvement in this respect.

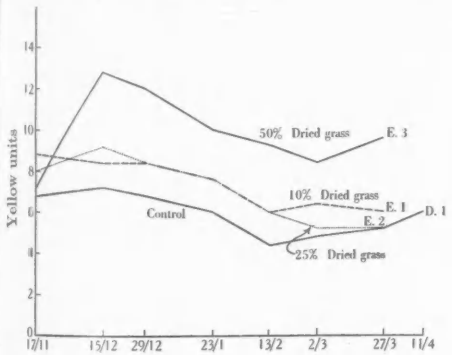

Fig. 1.

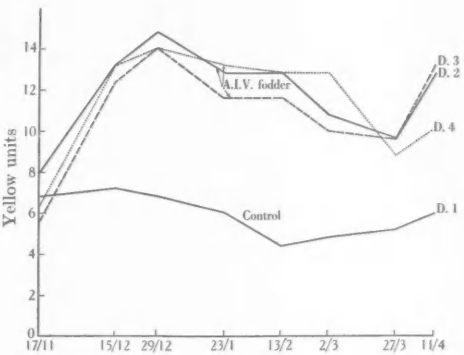

Fig. 2.

In the next experiment four pairs of cows were selected from the Shorthorn herd. One of these (group D1 *supra*) received an ordinary winter ration of hay, roots and concentrates, group D2 had a part of these replaced by 40 lb. of A.I.V. fodder, D3 by 70 lb. of A.I.V. fodder, whilst D4 received no purchased

foodstuffs but only A.I.V. fodder, hay and artificially dried grass, all four rations being of equivalent nutritive value (starch equivalent and protein equivalent).

A.I.V. fodder is produced by a modification of the ensilage process in which a forage crop—grass in this case—is treated with a dilute solution of mineral acids in such amounts that an acidity of p_H 3–4 is developed in the mass, which is stored in a suitable container to which the free access of air is prevented [Virtanen, 1933]. The temperature of the mass does not rise appreciably, and the carotene content of the green crop is retained in a large measure, whereas in the case of the warm fermentation process of ensilage, in which the temperature rises to the neighbourhood of 50°, most of the pigment is destroyed.

The test continued throughout the winter and the results are given in Table II in terms of the total yellow colours of the butters made from the milk.

Table II. *Total yellow colour of the butter from groups of Shorthorn cows on a normal winter ration, and on rations containing varying amounts of A.I.V. fodder.*

Stated as Lovibond units.

Week ending	D 1 Control	D 2 (40 lb. A.I.V.)	D 3 (70 lb. A.I.V.)	D 4 (70 lb. A.I.V. + dried grass)
17. xi. 32*	6·8	8·0	5·6	6·4
15. xii. 32	7·2	13·2	12·4	13·2
29. xii. 32	6·8	14·8	14·0	14·0
23. i. 33	6·0	12·8	11·6	13·2
13. ii. 33	4·4	12·8	11·6	12·8
2. iii. 33	4·8	10·8	10·0	12·8
27. iii. 33	5·2	9·6	9·6	8·8
11. iv. 33	6·0	12·8	13·2	10·0
Averages	5·8	12·4	11·8	12·1

* All four groups on control ration.

These results are also shown graphically in Fig. 2. The control group D1 produced butters which were pale in colour until the cows were turned out to pasture in April, when the colour value began to rise. The feeding of 40 lb. of A.I.V. fodder greatly increased the total yellow colour of the butter, whilst the 70 lb. of A.I.V. fodder were of equal value in this regard, but no better than 40 lb. Group D4 (A.I.V. fodder, artificially dried grass and hay only) showed no further advantage over the other two experimental groups. The rise in colour in April coincided with the advent of fresh grass.

Table III. *Carotene contents of A.I.V. fodder and artificially dried grass.*

Stated as mg./100 g. of the dry weight.

Date	A.I.V. fodder	Date	Artificially dried grass
23. xi. 32	52·2	6. xii. 32	47·9
13. xii. 32	33·9	20. xii. 32	38·0
11. i. 33	44·1	28. xii. 32	35·9
24. i. 33	38·1	11. i. 33	35·2
28. ii. 33	47·2	26. i. 33	40·1
21. iii. 33	40·8	6. ii. 33	34·4
—	—	10. ii. 33	46·9
—	—	16. ii. 33	36·0
—	—	24. ii. 33	26·6
—	—	3. iii. 33	26·7
—	—	10. iii. 33	28·4
—	—	16. iii. 33	34·5
Average	42·7	Average	35·9

Yellow units

The comparative effects of the rations of dried grass and of A.I.V. fodder in these experiments are shown in Fig. 3, where the group receiving 50 % of its concentrate ration as dried grass (E3) is compared with that receiving 40 lb. of A.I.V. fodder (D2) and with the control group (D1) in all of which the total nutrient values (S.E. and P.E.) of the rations were equal. The A.I.V. fodder has been more effective, but throughout the trial the cows on this diet were receiving a greater amount of dry matter and of carotene daily in the form of A.I.V. fodder than was fed in the form of artificially dried grass. The A.I.V. fodder supplied on the average 10 lb. of dry matter per head daily, whereas the dried grass supplied about 6 lb.

The carotene contents of the two foodstuffs were determined at intervals and the results are given in Table III.

In another experiment four pairs of Ayrshire cows were used (F1 to F4). The control group F4 received a normal winter ration similar to that fed to group D1 of the Shorthorns. To groups F1 and F3 were fed 40 lb. per head daily of A.I.V. fodder made with different acid mixtures, whilst group F2 received 40 lb. per head daily of a fodder made by the addition of a weaker acid mixture, sufficient to raise the acidity of the mass only to p_H 4·5, together with some molasses to stimulate a lactic acid fermentation.

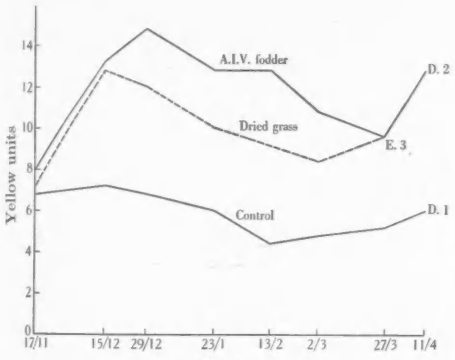

Fig. 3.

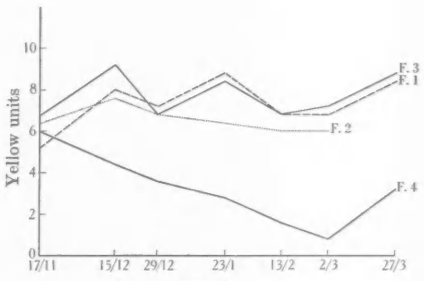

Fig. 4.

The results of this experiment are given in Table IV in terms of the total yellow colour of the butters.

Table IV. *Total yellow colour of the butter from groups of Ayrshire cows on a normal winter ration and on rations containing different types of acid-treated green fodder.*

Week ending	F 1 (A.I.V. fodder No. 1)	F 2 (Acid + molasses fodder)	F 3 (A.I.V. fodder No. 2)	F 4 Control
17. xi. 32*	5·2	6·4	6·8	6·0
15. xii. 32	8·0	7·6	9·2	4·4
29. xii. 32	7·2	6·8	6·8	3·6
23. i. 33	8·8	6·4	8·4	2·8
13. ii. 33	6·8	6·0	6·8	1·6
2. iii. 33	6·8	6·0	7·2	0·8
27. iii. 33	8·4	—	8·8	3·2
Averages	7·7	6·6	7·9	2·7

* All four groups on control ration.

The values are shown graphically in Fig. 4. The effect of the inclusion of a foodstuff rich in carotene is again apparent in the total yellow colour of the butter. The two A.I.V. fodders show some advantage in this respect over the fodder treated with a weaker acid mixture (fed to group F2) but in this case the fodder was made from grass of poorer quality than the other two types.

The carotene contents of the three fodders used are given in Table V; they account for the somewhat smaller effect obtained with the fodder made with a lower addition of acid. The supply of this latter fodder was exhausted in early March, and this group was then discontinued.

Table V. *Carotene contents of fodders fed to the groups of Ayrshire cows.*

(Stated as mg./100 g. of the dry matter.)

Date	F 1	F 2	F 3
13. xii. 32	56·7	33·3	64·6
11. i. 33	53·9	29·5	67·2
24. i. 33	50·4	25·2	56·1
9. ii. 33	38·5	47·4	42·8
21. ii. 33	28·8	35·6	59·5
21. iii. 33	41·4	—	58·2
Averages	44·9	34·2	58·1

An interesting point is the difference in level of the maximum yellow colour value of the butter in the Ayrshire groups as compared with the Shorthorns. With the former breed it scarcely exceeded 9 Lovibond units, whereas with the Shorthorns it rose to almost 15.

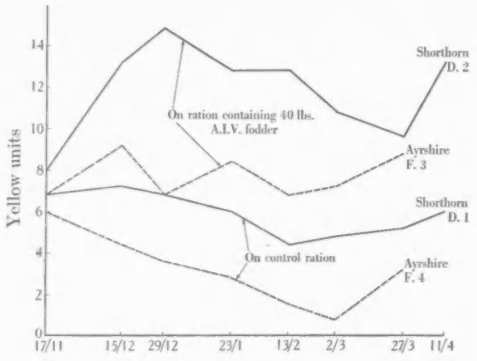

Fig. 5.

This difference with breed is shown clearly in Fig. 5, where the values for the two control groups, Shorthorn and Ayrshire, are plotted together with the values obtained when 40 lb. of A.I.V. fodder were fed (groups D2 and F3). It is noticeable that the Ayrshire butter lost its colour more rapidly and more completely than the Shorthorn on the same normal winter ration.

When a foodstuff rich in carotene was included in the ration the yellow colour of the butter was increased, but to a far greater extent with the Shorthorn cows. It seems that there is, for each breed, a "ceiling" value above which the depth of colour of the butter fat does not rise even when an excess of fodder rich in carotene is fed.

This ceiling value for the Ayrshire cows seems to be markedly lower than it is for the Shorthorns. Values obtained on the farm herds in June 1933, when the pasture was at its best, gave values of 15·2 Lovibond units for Shorthorn butter and 9·2 for Ayrshire butter, which agree well with the maximum values obtained on the A.I.V. fodder rations during the winter (14·8 and 9·2 respectively).

The difference in intensity of colour with breed has been shown by many workers, and it is generally known that the Channel Island breeds in Jersey and Guernsey produce a milk fat of higher colour than all the others, and that the Ayrshire falls below the other breeds in that regard. Wilbur *et al.* [1933] have shown that despite the difference in yellow colour, the vitamin A potency of the butter made from the milk of Jersey cows is no greater than that of Ayrshires. There are indications also from the values obtained from the butter of pure-bred Ayrshires that the vitamin A potency is similar to that of Shorthorn butter.

It would appear that the total yellow colour of butter from any one breed of cow is correlated with its carotene content and its vitamin A content, but that the depth of colour is no indication of the total vitamin A potency of the butter unless the breed from which the milk was obtained is known. A comprehensive experiment, including the major dairy breeds, is being conducted at present to obtain full data on this point.

Although the experiments were not designed to measure the effect of the various rations on the yield of milk, all yields were normal and the performance of all the cows was satisfactory.

Two trials of a different type were carried out at outside centres, the first at Dartington Hall in Devonshire[1], the other in Derbyshire. Thirteen South Devon cows were used at the former centre and eight Shorthorn cows in Derbyshire. All the cows at each centre were fed on a normal winter ration of concentrates for 5 weeks. They were all then changed over to an experimental ration containing 40 lb. of A.I.V. fodder per head daily, the nutritive value of the diet being the same as in the first period. After 5 weeks on this diet they were again changed back to the control ration, which was identical with that fed in the first period. The final period also lasted 5 weeks. The milk was sampled in the fifth week of each period, and butter samples were made and their total yellow colour was measured (Table VI).

Table VI. *Total yellow colour of the butter produced by two groups of cows on a normal winter ration and on one containing 40 lb. of A.I.V. fodder.*

Stated in Lovibond units.

	Period 1 Control	Period 2 A.I.V. fodder	Period 3 Control
South Devon cows	4·0	8·4	2·8
Shorthorn cows	2·0	8·4	2·4

The effect of the A.I.V. fodder on the total yellow colour of the butter is striking. The colour value is not so high as that obtained on 40 lb. of the A.I.V. fodder at Jealotts Hill, but it must be remembered that the experimental ration was fed for only 5 weeks and that the cows had been on a winter diet for 5 weeks, which had reduced the colour to a low level. It is a point of great practical interest that it is possible to raise the yellow colour of the milk fat so rapidly from a low level. The return of the colour to its previous low level

[1] A full account of this trial is being published elsewhere.

after reverting to the control ration for 5 weeks is indicative of the necessity for including a foodstuff rich in carotene in the diet of the dairy cow throughout the whole winter period if the colour of the milk and its quality are to be held at the high level which is so desirable from a nutritional standpoint.

II. THE CAROTENOID AND VITAMIN A CONTENTS OF THE BUTTER.

The preceding section of this paper describes the results obtained using as criterion the colour of the butter produced by the various experimental groups. The present section, on the other hand, gives the results of the spectroscopic examination of certain of the butters, with particular reference to the carotene, xanthophyll and vitamin A contents, determined by the method developed earlier [Gillam *et al.*, 1933]. The numerical results seen in Table VII correspond to the experiment described on p. 1077 (*cf.* also Table II).

Table VII.

				Constituents as mg./100 g. of dry butter			
Group	Ration	Date of milk sample	H_2O in butter	Carotene (1900 basis)	Xantho-phyll (1700 basis)	Vit. A by gross absorption 328 $m\mu$ (1600 basis)	Vit. A by absorption at 585 $m\mu$ in $SbCl_3$ colour test (2600 basis)
D 1	Control	16. i. 33	18·5	0·040	0·0186	0·292	0·222
		6. ii. 33	13·9	0·146	0·0144	0·298	0·157
		27. ii. 33	12·6	0·164	0·009	0·286	0·122
		20. iii. 33	17·9	0·025	0·0036	0·268	0·118
D 2	40 lb. A.I.V. fodder per cow per day	17. i. 33	16·0	0·607	0·065	0·63	0·480
		7. ii. 33	9·5	0·188	0·023	0·519	0·259
		28. ii. 33	12·7	0·347	0·039	0·494	0·196
		21. iii. 33	17·8	0·164	0·0134	0·407	0·281
		11. iv. 33	16·0	0·170	0·014	0·715	0·293
D 3	70 lb. A.I.V. fodder per cow per day	18. i. 33	12·8	0·543	0·063	0·372	0·294
		8. ii. 33	13·9	0·448	0·022	0·428	0·194
		1. iii. 33	17·0	0·325	0·0114	0·482	0·270
		22. iii. 33	19·1	—	0·026	0·430	0·169
		12. iv. 33	17·2	0·152	0·012	0·693	0·333
D 4	70 lb. A.I.V. fodder + 4 lb. dried grass per cow per day	18. i. 33	14·3	0·638	0·029	0·57	0·382
		9. ii. 33	12·7	0·362	0·043	0·575	0·341
		2. iii. 33	11·4	0·386	—	0·442	0·207
		22. iii. 33	12·4	0·388	0·0314	0·592	0·385
		13. iv. 33	15·6	—	—	0·578	0·241

The figures stated as the basis of calculation indicate the value of $E^{1\%}_{1\,\text{cm.}}$ taken as that of each of the pure substances. ($E = \log I_0/I$ for a 1 cm. layer of a 1 % solution, where I_0 = the intensity of the incident light and I = the intensity of the transmitted light at the wave-length of maximum absorption characteristic of each substance.)

The mean values for each of the groups over the whole period of the experiment are shown in Table VIII and clearly demonstrate the effect of the diet rich in carotene on the vitamin A and carotene contents of the butters.

A further experiment to test the value of A.I.V. fodder was carried out at Dartington Hall (Devonshire) using a single group of 13 pure-bred South Devon

Table VIII.

	Carotene mg./100 g. dry weight	Xanthophyll mg./100 g. dry weight	Vitamin A mg./100 g. dry weight U.V. values
D 1. Control	0·094	0·0114	0·286
D 2. 40 lb. A.I.V.	0·295	0·0308	0·553
D 3. 70 lb. A.I.V.	0·367	0·027	0·481
D 4. 70 lb. A.I.V. + 4 lb. dried grass	0·428	0·034	0·551

cows (see p. 1081). The results of the spectroscopic determinations are given in Table IX and, together with the evidence of the total yellow colour values given earlier, leave no doubt as to the value of A.I.V. fodder in the diet of the cow as a means of increasing the carotenoid and vitamin A contents of winter butter.

Table IX.

	First period	Second period	Third period
Ration	Control (5 weeks)	A.I.V. fodder (5 weeks)	Control (5 weeks)
Date of sample	25. i. 33	1. iii. 33	7. iv. 33
H_2O, %	13·7	12·1	12·6
Carotene, mg./100 g. of dry wt. (1900 basis)	0·122	0·311	0·129
Xanthophyll, mg./100 g. of dry wt. (1700 basis)	0·0081	0·0366	0·0148
Vitamin A, mg./100 g. of dry wt. By 328 $m\mu$ band (1600 basis)	0·183	0·405	0·240

The colour of cow's butter is known to vary with different breeds, but as the absence of yellow colour (or carotene) from butter does not necessarily imply the absence of the true vitamin A also, it was decided to examine a series of goat butters—which are noted for their chalky-white colour and low carotenoid content. Monthly samples were therefore obtained from one particular herd over the period June-December inclusive. The unsaponifiable matter was a much paler yellow than that of cow butter, and in chloroform solution it exhibited ill-defined maxima near 480 and 455 $m\mu$. As only a small fraction of the already weak yellow colour was extractable from light petroleum solution by methyl alcohol the amount of xanthophyll present must have been very small [Willstätter and Stoll, 1913]. The yellow colour remaining in the petroleum showed ill-defined absorption bands identical with those characteristic of the carotene fraction of cow's butter.

With antimony trichloride in chloroform the unsaponifiable matter gave a deep blue colour, much stronger than that to be expected from the small content of carotenoids. The blue solution exhibited two maxima at 612–618 $m\mu$ and 580–586 $m\mu$ characteristic of vitamin A, the presence of which was confirmed by the occurrence of an inflexion in the absorption curve of the chloroform solution near 328 $m\mu$.

Monthly samples (250 g.) were examined by the method applied to the cow's butter and the numerical results are given in Table X.

Vitamin A with antimony trichloride gives a blue colour exhibiting a strong absorption band at 617 $m\mu$ and a masked maximum close to 583 $m\mu$. The relative intensities of these bands are approximately 2 : 1 [Heilbron *et al.*, 1932; Carr and Jewell, 1933]. In cod-liver oil, however, the relative intensity of the 617 $m\mu$ band is considerably reduced presumably owing to the presence of certain inhibiting substances [Norris and Church, 1930]. Artificial inhibitors of this type, mostly decomposition products of proteins, have been described by

Table X. *Goat's butter.*

Date	H_2O %	Constituents expressed as mg./100 g. dry butter: Carotene* by light absorption at 460 $m\mu$	Vitamin A by 328 $m\mu$ band (1600 basis)	Vitamin A by 585 $m\mu$ band (in $SbCl_3$ blue) (2600 basis)
June	14·5	—	0·239	0·22
July	14·9	0·017	0·564	0·43
August	12·0	0·026	0·454	0·27
September	12·9	0·014	0·393	0·30
October	13·9	0·012	0·341	0·33
November	11·6	0·023	0·420	0·34
December	13·1	0·036	0·450	0·30

* Carotene values uncorrected for xanthophyll.

Table XI. *Comparison of goat's and cow's (pure-bred Shorthorn) butter.*

	Constituents expressed as mg./100 g. dry butter: Carotene	Vitamin A
Goat's butter. Mean values (June-December)	0·021	0·41
Cow's butter. Cows on ordinary winter diet (typical values)	0·15	0·28
Cow's butter. Cows on rich winter diet (typical values)	0·35	0·55
Cow's butter. Cows at grass (moderate summer values)	0·60	0·70

Emmerie *et al.* [1931], and the action of one of them—7-methylindole—on the vitamin A-antimony chloride blue colour has been examined fully [Morton, 1932]. The unsaponifiable matter of cow's butter must also contain these or similar inhibiting substances for with antimony trichloride in chloroform the intensity of the 617 $m\mu$ band is only the same or slightly more than that of the 583 $m\mu$ band and even the intensity of the latter band is reduced. This is also shown by the fact that the vitamin A values calculated from the intensity of this band are much lower than those obtained by the ultra-violet absorption at 328 $m\mu$. The distinct nature of this inhibition will be seen by a comparison of the vitamin A values measured by the two methods (Table VII). The inhibition of these absorption bands in the blue antimony trichloride solution of the unsaponifiable matter of butter is probably due to the same cause as that which gives the low blue colour values (Lovibond) on butter fat reported by Booth *et al.* [1933], who find the amount of inhibition to vary seasonally. In the samples of the unsaponifiable matter of goat's butter we find that although both absorption bands are reduced in intensity the inhibition is not so marked as in the case of cow's butter. This is shown by the fact that the vitamin A values of goat's butter, calculated from the intensity of the 585 $m\mu$ absorption band, do not differ quite so much as in the case of cow's butter from those obtained by the ultra-violet absorption method (Table X).

Summary.

The effect of a ration containing artificially dried grass on the quality of the milk of Shorthorn cows was tested. When 10 or 25 % of the concentrated foodstuffs fed in the production ration were replaced by artificially dried grass no appreciable advantage in depth of yellow colour of the butter-fat was noted, as compared with a control group on a normal winter ration. The inclusion of

50 % of artificially dried grass in the concentrated ration, however, caused a distinct improvement in the yellow colour of the butter fat.

The inclusion of A.I.V. fodder in the winter ration of Shorthorn cows raised the yellow colour of the butter-fat to a high level, comparable with that obtained on best pasture. The A.I.V. fodder was fed at two levels, 40 lb. and 70 lb. per head daily. The larger amount showed no advantage, either in colour or vitamin A content of butter fat, over an allowance of 40 lb. per head daily.

The feeding of A.I.V. fodder increased the carotene and xanthophyll contents of the resulting butter to about three times those of a similar control group on a normal winter ration. The content of the true vitamin A was approximately doubled at the same time.

The feeding of A.I.V. fodder to Ayrshire cows raised the yellow colour of the butter-fat to a level well above that from milk produced by a group on a normal winter ration.

The difference in the depth of yellow colour of butter fat as between breeds, is well marked in the case of Shorthorn and Ayrshire cows. On a normal winter ration deficient in carotene, the yellow colour of the butter fat of Ayrshire cows fell more rapidly and to a decidedly lower level than was the case with Shorthorn cows. There seems to be, for each of these two breeds, a "ceiling" value above which the colour intensity and vitamin A content of the butter fat do not rise, even when an excess of carotene-rich fodder is fed.

The feeding of 40 lb. of A.I.V. fodder in the ration raised the yellow colour of the butter fat of Shorthorn cows to a high "ceiling" level, whereas with the Ayrshire the "ceiling" value was much lower, though the amount of carotene supplied in the ration was similar for both breeds. In spite of this difference in depth of colour, it appears that there may be little difference in the content of true vitamin A in the butters from the two breeds.

Samples of goat's butter were found to be very similar to cow's butter in their content of the true vitamin A, although they were almost devoid of colour; the carotene content was less than 5 % of that of the butter from cows at grass. Thus the colour of goat's butter cannot be used as an indication of its growth-promoting activity in comparison with cow's butter, since in goat's butter most of this activity is due to the presence of colourless preformed vitamin A and not to carotene.

The thanks of the authors are due to the Research Council of Messrs Imperial Chemical Industries, Ltd. for a grant which has defrayed part of the expenses of this work, to Mr W. S. Ferguson and other members of the staff there for their assistance in the work carried out at Jealotts Hill, and to Mr H. J. Page, under whose direction that work was carried out.

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CXLVIII. AN ANALYTICAL STUDY OF LIESEGANG RINGS.

By EDWIN BURNHOPE HUGHES.

(*Received March 12th, 1934.*)

So much has been written on Liesegang rings, and so many theories or variants of theories proposed, that apology may be expected for further addition, though perhaps the investigation of the phenomenon mainly by the application of quantitative analysis may be regarded as some justification. The literature of this subject has been liberally referred to in most of the papers published thereon in recent years, including an exceptional comprehensive bibliography by Hedges [1932] and it is accordingly considered unnecessary to repeat such references here, except in those instances where specific mention is essential.

In order to make clear the trend of the results of this investigation, it may be stated that they lead to the conclusion that, when diffusion of one electrolyte into a solution of another of lower equivalent concentration (generally contained in a gel) causes formation of a precipitate, the occurrence of this precipitate in band formation depends essentially on the following factors:

(1) The gradient of the concentration of the entering diffusing reacting salt (precipitating ion, A) and the resulting gradient, at the precipitate front, of the concentration of the reacting ion (B) contained in the medium; and on the removal of ion B in the neighbourhood of the precipitate by the advent of more of ion A till forward diffusion of electrolyte A again proceeds normally.

(2) On the rapid collection of the separated precipitate into a more closely defined zone than when first separated.

(3) On the solubility of the precipitate and on some factor explaining sudden separation of precipitate, which is taken to be metastable solubility.

Factors (2) and (3) are essential conditions to the extent that on them depend the character of precipitate and the conditions for (1) requisite to control the formation of the precipitate in banded form or as continuous precipitate.

Furthermore, the primary importance of the movement of the electrolytes towards each other is shown by the fact that banded precipitation can be obtained when this movement of ions is produced under the influence of a superimposed E.M.F., *i.e.* by the application of electrolysis [Hughes, 1928].

None of these factors is new in its application to the explanation of the formation of Liesegang rings; they have been proposed and referred to, to a greater or less extent, in many notable papers, but in this communication they are submitted as a conclusion from the experimental evidence recorded here. There are other factors which may be of importance in certain instances only—such as adsorption, and coagulation of the precipitate by the entering electrolyte (or one of its ions)—but these factors are to be regarded as of minor importance only and not as essential causes of banded formation of precipitate.

A theory of the formation of Liesegang rings follows the description of the experimental work.

A. Diffusion.

The process of diffusion of an electrolyte into a gel follows Fick's law of diffusion, and the mathematical expressions for the concentration gradient and the rate of penetration have been developed and verified experimentally by a number of workers, *e.g.* Morse and Pierce [1903], Stiles [1920], Stiles and Adair [1921], Adair [1920]. A very valuable mathematical and theoretical study of diffusion in respect of rhythmic precipitates is that of Fricke [1923]. The effect of temperature on rate of diffusion is considerable: Stiles [1921] states that increase of temperature from 0° to 20° increases the diffusion coefficient about 1·76 to 2·2 times, according to the salt.

A I. *The passage of water into or from the gel.*

The possibility of the movement of water into the gel from the outside or *vice versa* is generally ignored though, for example, Hatschek [1922], Davies [1922] and Steopoe [1924], have suggested that alteration of the water content of the gel may have some bearing on the formation of Liesegang rings. Accordingly it seemed worth while, first of all, to ascertain if such action takes place to an appreciable extent.

Diffusion was caused to take place for about 100 hours from a weighed solution of a salt of known concentration into a weighed tube of gelatin gel, after which the solution and gel were separately weighed and analysed for salt in the solution and salt and gelatin solids in the gel. Similar tests were made with a salt in the gel diffusing out into the water. The relative amounts of aqueous solution and of gel were varied. The materials used were: NaCl solution and gelatin gel and agar gel; K_2CrO_4, also $AgNO_3$ into plain gelatin gel and into the gel containing the other salt; water into gelatin gel containing NaCl. Results showed that the movement of water only occurred to a negligible extent, generally about 0·1 to 0·3 g., being to the solution of higher osmotic pressure, and always only from near the surface of the gel. It is concluded that, in connection with diffusion from solutions into gels, the movement of water is very slight and is negligible.

Actually if two reacting salts are separately contained in gels in contact, diffusion from one gel to the other with formation of a banded precipitate can occur.

A II. *Analysis, with respect to the entering electrolyte, of gels in which diffusion has taken place with formation of precipitate (continuous or banded).*

In general, a quantity of standardised solution of one electrolyte was mixed with liquefied gel of concentration double that finally required and of half the required volume, and the mixture then made up to volume with distilled water. A portion was taken for analysis and the remainder filled into corked glass tubes of about 2·5 cm. diameter and suitable length. When the gel had set firm, it was attached by rubber tubing to the neck of a flask (300 ml.)[1] containing the intra-diffusing electrolyte, and then placed vertically, with flask inverted, and the diffusion allowed to proceed at constant temperature to the required extent[2]. The flask was then disconnected,

[1] In general sufficient volume of reagent to ensure only the slightest diminution of concentration during diffusion.

[2] It was proved that this method gave entirely satisfactory results, and that there was no entry of the diffusing liquid itself in experiments recorded here.

With gelatin gels or silicic acid gels no entry of diffusing liquid ever occurred; with agar gels it was found that this trouble could be avoided by filling the hot agar solution (at about 95°) into hot tubes taken direct from a steam-oven, the contraction of the agar from the glass, on setting, being thus avoided.

free liquid carefully removed from the surface of the gel with filter-paper, and the gel removed from the tube by one of the following methods.

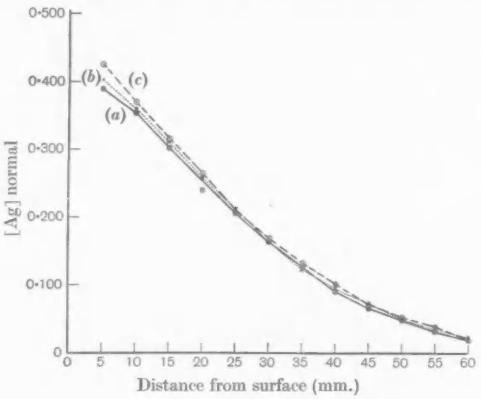
(*a*) The tube used had been first carefully split in two, the halves bound together with rubber bands and the cut edges waxed over before filling. Afterwards, when the gel was required for analysis, the wax was scraped off the tube and the two portions carefully separated, leaving generally a clean undamaged cylinder of gel.

(*b*) Procedure (*a*) was not always successful; occasionally a gelatin gel would adhere to the tube in places, spoiling the experiment. The method finally adopted, which avoided the use of split tubes, was to remove the gel by gently pushing a long stiff thin wire along the inside of the tube, closely in contact with the glass, and then, while one person held the wire firmly, another gently rotated the tube so that the wire was always against the glass. The gel was so loosened and could then be blown out of the tube.

(*c*) Agar gels contract on setting, so that all that is necessary is to cut round the top edge of the gels (where contact had purposely been made sound to avoid entry of diffusing liquid) and then to blow the gel out of the tube.

The gel was then sliced with a razor blade into portions of suitable thinness, and these were placed at once in small covered weighed beakers and weighed, and the slices were then analysed. Generally the thickness of each slice was not measured directly, but calculated from its weight in relation to the total weight of the slices, the total length of the gel before cutting being noted. Distances were calculated as being to the middles of slices, and in recording analytical results these are given as if the slices were of uniform composition. In the graphs the concentration of reagent in a slice is given as at the middle point, or sometimes shown (for bands and clear spaces) as being equal throughout the slice.

Graph 1 (curves *a*, *b*, *c*) shows the concentration of silver in the gel after diffusion thereinto of $N/2$ $AgNO_3$ solution (*a*) into plain 5 % gelatin gel; (*b*) similar gel containing $N/500$ K_2CrO_4; (*c*) gel containing $N/500$ Ag_2CrO_4.



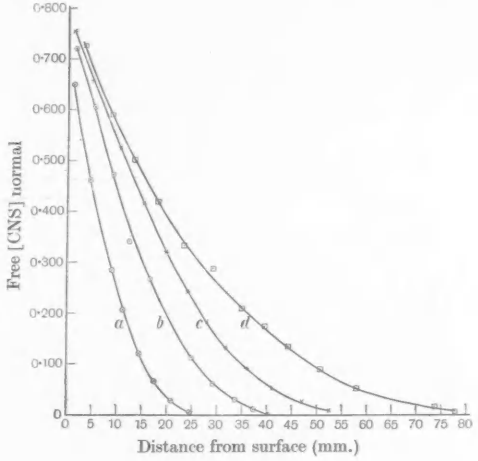
Graph 1.

(*a*) ⊗—⊗ 5 % gel only. (*b*) ×----× 5 % gel + $N/500$ K_2CrO_4.
(*c*) ⊙---⊙ 5 % gel + $N/500$ Ag_2CrO_4.

These results show that in presence of Ag_2CrO_4 in solution or of very dilute K_2CrO_4 in the gel, the concentration curve of the intra-diffused $AgNO_3$ is of the same type as for the diffusion into the plain gel. (*b*) and (*c*) show slightly higher concentration of Ag than (*a*) due to the Ag precipitated by the K_2CrO_4 and to the Ag_2CrO_4—but the amount of Ag so held is not constant along the tube,

i.e. is not always $N/500$, this being due to some movement of the contained K_2CrO_4 (and Ag_2CrO_4) towards the entering silver. This effect is not very noticeable for such dilute K_2CrO_4 and, moreover, the rings are not shown separately; clear evidence of this movement is given in results described later.

The inference, from the deduction and observation of Morse and Pierce [1903] that the relationship $d/\sqrt{t}$ applies during ring-formation, is that the diffusion proceeds in accordance with Fick's law all through the precipitate formed, and that the concentration of the uncombined entering diffusing ion should therefore give an ordinary diffusion curve. Results such as those shown in Graph 1, comparing diffusion in absence and in presence of a precipitating ion, indicate that this is the case. Consequently, subtraction of the concentration of other ion (contained in the gel) from the equivalent of the concentration of diffusing ion at the same point should give a regular diffusion curve for the differences so obtained, *i.e.* for the "free" ion[1]. This procedure has been followed in considering the analytical results which follow. A simple case is where the "free" ion can be determined directly without having to determine each of the reacting ions and subtract; for example, Graph 2 (curves *a*, *b*, *c* and *d*), which expresses the results of analysis after diffusion of ammonium thiocyanate into 5 % gelatin containing $N/70$ silver nitrate for 19, 43, 67 and 139 hours. The tubes and amounts of reagents were identical, so that the results can be taken to represent the concentration in one tube after these different periods of diffusion.



Graph 2.

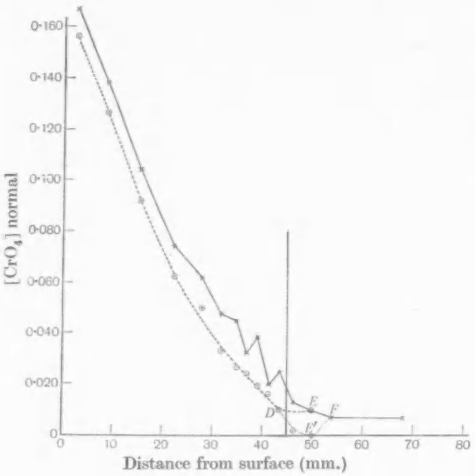
a After diffusion for 19 hours. *b* After diffusion for 43 hours.
c „ „ 67 „ *d* „ „ 139 „

It will be seen that the curves, which are for "free" [CNS], are satisfactory diffusion curves. They also show clearly the flattening of the gradient as diffusion proceeds further. The results obtained for [Ag] (not shown) indicate that there is also a diffusion gradient of the [Ag] in the gel towards the precipitate front.

[1] The term "free," in considering the ion or the salt, is applied to the amount or concentration of it which is not combined or associated with the other ion with which it forms a precipitate.

When separate bands and "clear" spaces are analysed, results have to be interpreted as to the centre of the bands, and to inexactness from this must also be added some inaccuracy resulting from not being able to cut slices exactly to the edge of a band, even if sharply defined, and also that resulting from the double experimental error which may be involved in subtraction. Nevertheless, the results obtained[1] provide satisfactory evidence of the regular diffusion of the "free" ion. Examples are given in Graphs 3, 4, 5 and 6.

Diffusion of M/*10* K_2CrO_4 *into 5 °/₀ gelatin containing* N/*100* $AgNO_3$. *Analysis after diffusion for 121 hours* (Graph 3). The general form of the curve for "free" [CrO_4] is in accordance with diffusion, as expected. The "free" curve is joined



Graph 3.

× —— × Total [CrO_4]. ⊙ - - - ⊙ Free [CrO_4].

to the total curve at E because from there onwards the CrO_4 is in presence of Ag without any precipitate having been formed. $DE'F$ would represent the conditions when precipitation for a band occurs at (or about) E'.

Diffusion of M/*5* $K_2Cr_2O_7$ *into* N/*100* $AgNO_3$ *in 5 °/₀ gelatin* (Graph 4). Analysis was made of separate bands and spaces from 103 mm. below the surface, where the bands were wide enough apart for separation. The last ring was incompletely formed. The time of diffusion was 52 days, after which the action had practically come to a standstill although there was abundant $AgNO_3$ remaining, this having been assured by using a long tube of gel fitting into a flask of the gel.

With $AgNO_3$ as the internal electrolyte the diffusion of $K_2Cr_2O_7$ into the gel results in the formation of Ag_2CrO_4[2] (not $Ag_2Cr_2O_7$), according to the equation

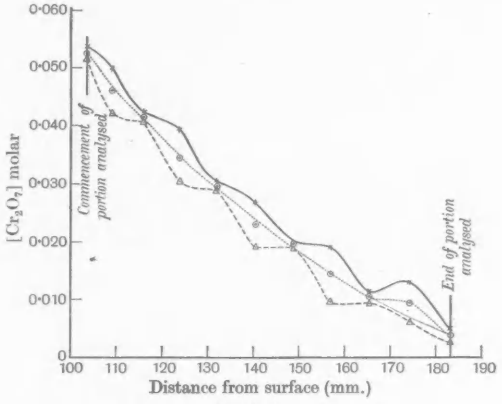
$$H_2O+K_2Cr_2O_7+4AgNO_3=2Ag_2CrO_4+2KNO_3+2HNO_3.$$

The "free" [Cr_2O_7] is calculated accordingly, and the curve is seen to be of a satisfactory character, except towards the end of diffusion; here, however, the

[1] In none of these examples is allowance made for the solubility of the precipitate; such allowance would not affect the shape of the "free" curve.

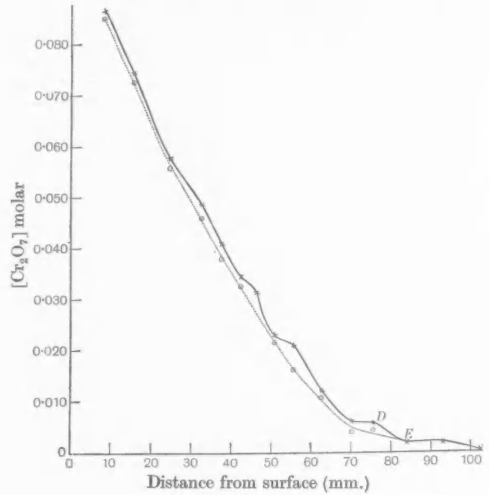
[2] Davies [1922], also confirmed (in addition to Graph 4) by tests applying Pringsheim's rule and by the colour and nature of the precipitate.

last ring was ill-formed and indefinite, there being some other separation of precipitate, near the band, on the glass tube. For comparison, the curve for "free" $[Cr_2O_7]$ assuming formation of $Ag_2Cr_2O_7$, is also given. $K_2Cr_2O_7$ is more suitable than K_2CrO_4 for obtaining bands for analysis, as the spacing is wider; this can be explained by the lower effective concentration of the $AgNO_3$ when $K_2Cr_2O_7$ is used with it.



Graph 4.

× —— × Total $[Cr_2O_7]$. ⊙- - - ⊙ Free $[Cr_2O_7]$ (formation of Ag_2CrO_4).
△– – – △ Free $[Cr_2O_7]$ (if $Ag_2Cr_2O_7$ were formed).



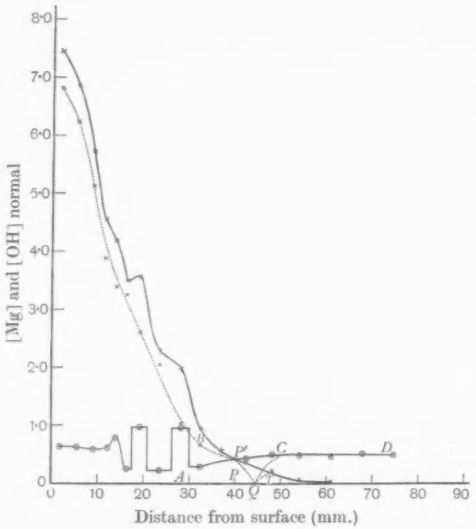
Graph 5.

× —— × Total $[Cr_2O_7]$. ⊙- - - ⊙ Free $[Cr_2O_7]$.

Diffusion of 0·1 M *$K_2Cr_2O_7$ for 257 hours into 0·0075* N *$AgNO_3$* (another example of the same type) (Graph 5). The "free" $[Cr_2O_7]$ curve is run into

the total [Cr_2O_7] curve between points D and E for the same reason as stated with reference to Graph 3. The gradient of the concentration of the Ag has become very flat from E onwards, and progress of diffusion of both $K_2Cr_2O_7$ and Ag has practically ceased (as occurs relatively early when a salt in dilute solution diffuses, with formation of precipitate, into one very dilute).

Diffusion of ammonia (solution of sp.gr. 0·880) into N/2 $MgCl_2$ contained in 1 % agar gel. Analysis after diffusion for 48 hours (Graph 6). At the moment of cutting the gel the last actual precipitate was at A. The "free" [OH] curve should then pass on to the total [OH] curve at P' after which OH is "free" in the presence of Mg. Apparently at about PP', say at Q, precipitation is due. The "free" [OH] should there drop to a low value as BQT; "free" [Mg] would drop from P' to a low value at Q, so that its curve would have a gradient from Q into CD, say at C.



Graph 6.

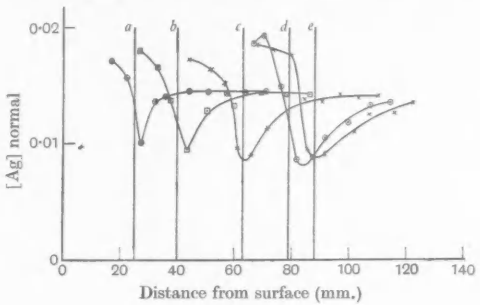
× —— × Total [OH]. ⊙ —— ⊙ Total [Mg]. × - - - - × Free [OH]. × ····· × Free [Mg].

In the examples just given it will be noted that the entering ion penetrates some distance into the gel beyond the last precipitate (band) without forming precipitate. This distance of penetration obviously has relation to the spacing of the bands; it is, in fact, a governing condition. The flatter the curve the further will the ion penetrate without forming precipitate; accordingly a concentrated solution (of the entering ion) will tend to give continuous precipitate, as, its gradient being steep, it will have little penetration beyond the precipitate front, and a dilute solution will give bands—if other conditions are also suitable. When the concentration curve of a concentrated solution becomes sufficiently flat owing to time and distance of diffusion, then the precipitate may change from continuous to band-formation.

The concentration of the ion contained in the gel also has its effect on band-formation, and accordingly the gradient of the concentration of the internal ion has been examined.

A III. *Analysis, with respect to the internal electrolyte, after diffusion has taken place with formation of precipitate (continuous or banded).*

Curves *a*, *b*, *c*, *d* and *e* in Graph 7 show results of analyses of the gel in the neighbourhood of the precipitate front after diffusion of *N* K_2CrO_4 into 0·0143 *N* $AgNO_3$ in 5 % gelatin in similar tubes for different periods (16, 42, 112, 161, 209 hours). These conditions give close but separate bands[1].



Graph 7.

The vertical lines represent the precipitate front (as nearly as it could be cut) for the tubes *a*, *b*, *c*, *d*, *e*. The results show clearly:

(1) that the Ag, in amount, has advanced very considerably towards the incoming K_2CrO_4. This is to be expected from the gradient of the [Ag] at the precipitate front, down which the Ag must move.

(2) The gradient flattens with the age (and distance) of the diffusion of K_2CrO_4. (It is the right-hand slope of the curve which is to be considered.) This change in the gradient of the internal electrolyte results in the widening of spaces between rings; it is dependent on the rate of diffusion of the entering ion. If the conditions of diffusion give continuous precipitate, or rings so closely arranged as to be merely denser zones with precipitate between, then the gradient of the contained ion shows but little alteration as diffusion proceeds.

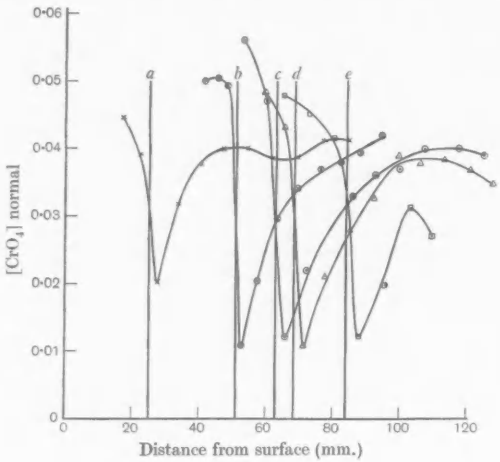
Curves *a*, *b*, *c*, *d* and *e* in Graph 8 illustrate this in the case of diffusion of 0·5 *N* $AgNO_3$ into 0·04 *N* K_2CrO_4 in 5 % gelatin for 24, 120, 172, 216, 312 hours. In tube *e* the CrO_4 remaining uncombined is much reduced in amount.

Graphs 9 and 10 are of interest as showing results when either concentrated $AgNO_3$ or K_2CrO_4 diffuses into the other also rather concentrated. Such conditions result in continuous precipitate.

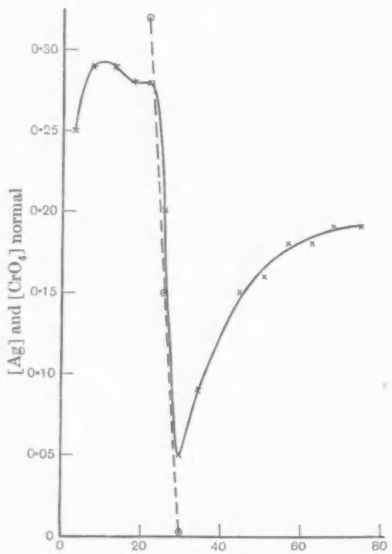
The observations of interest are:

(1) The steepness of the curve of the concentration of the entering electrolyte. This is a condition for formation of continuous precipitate. This steepness is due, first to the high concentration of the outside electrolyte and, secondly, to the somewhat high concentration of the internal electrolyte which has the

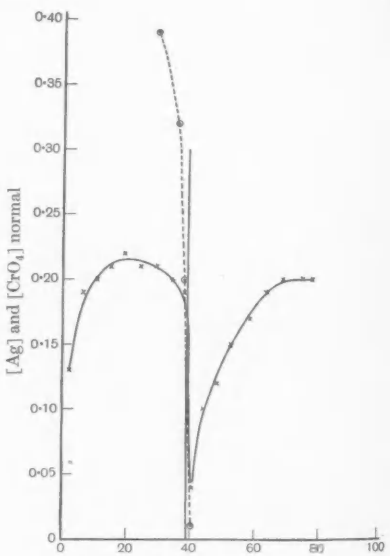
[1] For the purpose of obtaining clear spaces and bands of Ag_2CrO_4 for analysis, it is preferable to have $AgNO_3$ as the internal electrolyte.



Graph 8.



Graph 9.



Graph 10.

Graph 9. Diffusion of N K_2CrO_4 into 0·2 N $AgNO_3$ in 5 % gelatin for 93 hours.
× —— × Total [Ag]. ⊙— —⊙ Total [CrO_4].

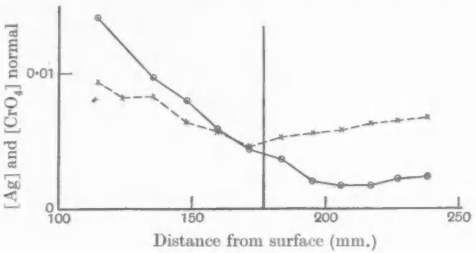
Graph 10. Diffusion of N $AgNO_3$ into 0·2 N K_2CrO_4 in 5 % gelatin for 70 hours.
× —— × Total [CrO_4]. ⊙- - - -⊙ Total [Ag].

effect of slowing down the diffusion of the entering electrolyte, *i.e.* making k, of $d/\sqrt{t}=k$, much smaller. Thus, for example, diffusion of N $AgNO_3$ into 5 % gelatin containing (*a*) no added salt, (*b*) $N/100$ K_2CrO_4, (*c*) $N/50$ K_2CrO_4, (*d*) $N/10$ K_2CrO_4, (*e*) $N/5$ K_2CrO_4, gave results as follows for periods up to 112 hours: k (cm.-hours) *a*, 0·74; *b*, 0·68; *c*, 0·64; *d*, 0·46; *e*, 0·38.

(2) The gradient of the concentration of the internal electrolyte, at the precipitate front, is also steep. The consequence is that the steepness of both gradients ensures that a very slight advance of the entering electrolyte will provide sufficient of it in the internal electrolyte to produce the amount required for the metastable solubility[1] of the precipitate, which is therefore continuous.

(3) The internal electrolyte has moved up the tube very considerably.

Graph 11 gives the results of conditions of diffusion of the extreme opposite of the last.



Graph 11.

⊙——⊙ Total $[CrO_4]$. ×---× Total [Ag].

Rather dilute K_2CrO_4 ($N/10$) had been diffusing into very dilute ($N/100$) $AgNO_3$ in 5 % gelatin contained in a long tube which was continued into a flask of the $AgNO_3$ in gel, so that there was abundance of each electrolyte. For 21 days bands continued to form, after which time diffusion had become so slow that in a further 38 days only a trace more of precipitate had formed, and this only on the wall of the tube.

The analytical results show that after a depth of 170 mm. the gradient of $[CrO_4]$ was so slight that the CrO_4 diffused for 67 mm. further without rising above 0·002 N, and that similarly the gradient of the [Ag] was so flat that even at 60 mm. from the precipitate front the concentration was only 0·006 N, hardly enough to cause precipitation. Under such conditions diffusion of both electrolytes virtually comes to a standstill, and it is probable that, as the real metastable solubility is not reached, some partial separation of precipitate occurs, for example, on the walls of the tube—as observed; this would also probably account for the slight rise in $[CrO_4]$ at the end of the diffusion.

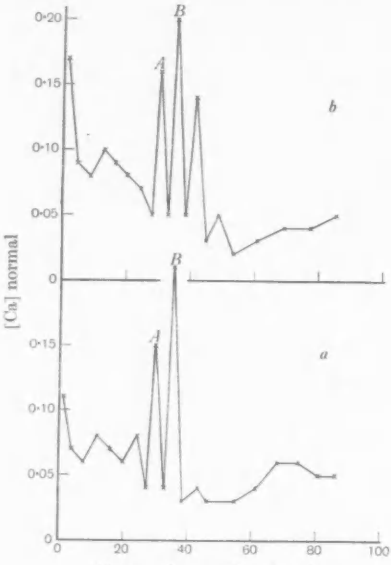
A IV. *Permanency of amount of precipitate in fully formed bands.*

The results just considered all show that the concentration of the internal electrolyte falls to a small quantity (actually, at the lowest point, the solubility of the precipitate), in the immediate neighbourhood (front) of the precipitate, and accordingly it is to be expected that this electrolyte does not pass appreciably further towards the entering ion. This implies that a band, once fully formed, does not subsequently acquire any increase in precipitate caused by

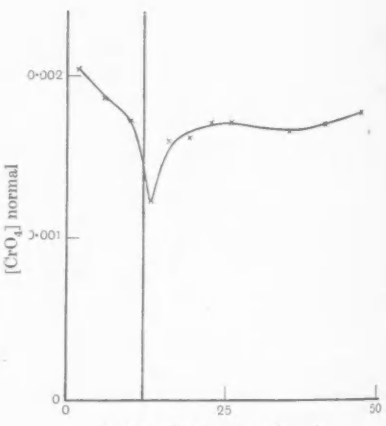
[1] See p. 1099.

the reacting electrolytes, though for some time after the formation of a band, until fully formed, there will be some considerable intensification of the precipitate forming it, due to collection on to it of precipitate from the adjacent "clear" spaces (p. 1100), and also due to the reduced solubility of the precipitate, if this is less soluble in higher concentrations of entering ion, *e.g.* Ag_2CrO_4 in $AgNO_3$; the reverse, *i.e.* increased solubility, also occurs, *e.g.* AgCNS in NH_4CNS. The following is an example showing that the bands, once completely formed, continue, in the main, unchanged in intensity.

Curves *a* and *b* in Graph 12 show the distribution of calcium in two identical tubes after diffusion of 0·166 *M* Na_2HPO_4 into a 1 % agar gel containing 0·066 *N* $CaCl_2$ for (*a*) 141 hours and (*b*) 237 hours. If one of the curves is superimposed on the other it will be seen that the peaks *A* and *B* in the one fit almost exactly on the same two peaks of the other.



Graph 12.



Graph 13.

A V. *Diffusion of silver chromate.*

A particularly interesting example of analysis after diffusion is that showing results of diffusion of a solution[1] of silver chromate contained in a gelatin gel, towards incoming silver nitrate, when bands of precipitated silver chromate are formed.

This precipitation has been described by Williams and MacKenzie [1920] and by Hedges and Henley [1928], the former considering it to be evidence of diffusion of silver chromate, and the latter as periodic coagulation or periodic salting out; both of these actions occur, but the results here given particularly

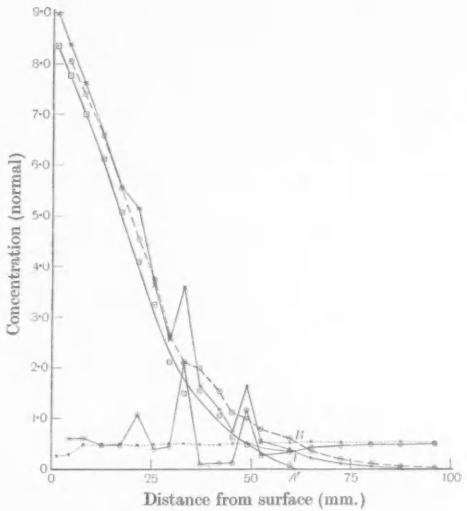
[1] Experimental evidence as to the solution-state of Ag_2CrO_4 when yellow has also been given by Bolam and Mackenzie [1926] and Desai and Nabar [1931; 1932].

confirm that silver chromate in solution does diffuse and band formation is to be attributed to this.

In this experiment, ash-free gelatin prepared by the method of Loeb [1922] was used in order to avoid the interference due to chlorides and phosphates; in such gelatin the solubility of Ag_2CrO_4, as measured by the maximum amount which still gives only a yellow colour (no tinge of red), is very low, and in consequence the concentration of Ag_2CrO_4 used was only $N/500$. Diffusion was for 4 hours only. Such a brief period was necessary in order that the gradient of the $[Ag_2CrO_4]$, which at most can only be short, shall be as steep as possible, this being governed by the rate of diffusion of the entering $AgNO_3$.

A VI. *The unprecipitated electrolyte of the reaction.*

Results so far given have been concerned solely with the precipitating ions and not with the unprecipitated product (or ions) of the reaction. A convenient example of ring-formation for this full analysis is provided by the diffusion of ammonia into magnesium chloride in agar gel which other workers [Ostwald, 1925; Hedges and Henley, 1928; Fricke and Suwelack, 1926] have found particularly suitable (Graph 14).



Graph 14.

× —— × Total [OH]. ⊙- - -⊙ Total [NH_4]. ⊙ —— ⊙ Total [Mg].
× - - - × Total [Cl]. ⊡ —— ⊡ Free [OH].

Consider the concentration curves for each ion after diffusion for a period. The [Mg], ion contained in the gel, will give a curve having the usual gradient towards the last formed precipitate and with peaks representing the bands of $Mg(OH)_2$. The $[NH_4]$, entering ion, will give a diffusion curve representing mainly NH_4OH and some as NH_4Cl—but on the whole it should be an ordinary diffusion curve without peaks due to any local concentration. The [OH], entering ion, will give a diffusion curve almost coincident with the $[NH_4]$ curve except

for peaks showing the bands of $Mg(OH)_2$. The "free" [OH] will give a concentration curve very similar to that of the $[NH_4]$, separated only by the equivalent of the [Cl].

There remains the [Cl], ion contained in the gel. It has not entered into any real combination or diffusion. It should therefore show no peaks, and, as it was originally uniformly distributed throughout the tube it should remain so—without any diffusion, except perhaps on account of NH_4Cl when formation of $Mg(OH)_2$ takes place, which would diffuse in both directions from the zone of the reaction; there would be also some diffusion out into the NH_4OH in the reservoir. Consequently the [Cl] curve should be, in the main, a horizontal line. A typical example (diffusion of ammonia, sp. gr. 0·880, into 0·5 *N* $MgCl_2$ in 1 % agar gel), Graph 14, shows that these conditions are fulfilled.

These results, which are all analytical determinations, also indicate another matter of interest, namely that, up to the forefront of the precipitate, there is diffusion of NH_4OH as molecular diffusion, but that from that zone the diffusion becomes ionic. At the front of the precipitate the action occurring is the union of OH and Mg, with the consequence of diffusion of NH_4 forward in excess of the OH, and diffusion of Mg towards the OH, always, of course, so that $[Mg]+[NH_4]=[OH]+[Cl]$. For example, note the two points *A* and *B* where [OH] and [Mg], and $[NH_4]$ and [Cl] are respectively equal.

The point *A* has also another significance. It will be noted that the curve for "free" [OH] is joined on to that for total [OH] at the point *A*, on the assumption that, from about there onwards, all the OH is free.

Fricke and Suwelack [1926] have published analyses and curves of a number of examples of diffusion of NH_4OH into $MgCl_2$ contained in agar—chiefly from the last ring (or 2 rings) forward into the $MgCl_2$ and have shown therefrom the dependence of ring-formation on the ion activity product.

Summary of the results and conclusions from the analytical examination of banded and continuous precipitate.

1. The results recorded indicate the predominant effect of conditions of diffusion in governing the resulting form of the precipitate, *i.e.* whether it is banded or continuous. The theoretical relation of gradient of entering ion to spacing of the bands is described later.

2. They show also that the reaction is limited to the precipitate front with, as suggested by the results of microscopic examination of rapidly forming bands (as under the influence of a superimposed E.M.F.), some condensation and growth of the precipitate immediately it is liberated and some subsequent clearing of spaces due to reduction in solubility caused by the increasing concentration of the entering ion.

3. At the precipitate front is a gradient of concentration of the ion contained in the medium (gel). This gradient obviously has an effect on the spacing of bands, and is dependent on the concentration of this ion in the medium and also on the gradient of the concentration of the entering ("free") ion.

Before these conclusions can be embodied clearly in a theoretical explanation of the formation of Liesegang rings, the specification of some other conditions becomes necessary, namely some factors which will account for the sudden liberation and development of precipitate. These factors are not essential only to banded precipitate; they will function when the precipitate is continuous. The conceptions which seem best to meet these requirements are: (*a*) metastable solution and (*b*) growth of precipitate particles at the expense of the smallest

[Jablczynski *et al.* 1923], and (*c*) removal of contained ion in the zone of the precipitate (as described on p. 1103). Some brief reference is therefore given to these.

B. METASTABLE SOLUTION AND DEVELOPMENT OF PRECIPITATE.

Much of the published work with reference to production of banded precipitates is a record of the precipitates which will, under specified conditions, form in bands. It is generally agreed that almost any substance which is relatively insoluble can be obtained in band-formation if the conditions are satisfactory [see Hedges and Henley, 1928; Morse, 1930]. Notable among these conditions is the degree of solubility of the precipitate in the medium, which is generally a gel, but which may also be simple aqueous solution. The solubility can be so altered by choice of the medium or by addition of a small quantity of some foreign substance that bands can be obtained for almost any material which forms as a precipitate by double decomposition. This is well recognised [see Bradford, 1920; Morse, 1930].

Supersaturation and metastable solution.

Banded precipitates can only be formed in conditions which are actually ideal for the occurrence of supersaturation, an important condition being undisturbed diffusion (with or without a gel). In such circumstances there is none of the solid phase present where precipitation occurs, and no disturbance to cause the solubility to be limited to the true solubility. On the other hand the sparingly soluble salt formed does not remain indefinitely in solution but appears to be released at some limiting value, indicating a metastable limit. Though such an occurrence may not be capable of direct proof[1], it seems to be a necessary assumption in accounting for the formation of precipitate in bands. The arguments in favour of this opinion have been put forward by many workers, notably Morse and Pierce [1903] who state:

"We have shown by quantitative measurements that in the formation of silver chromate in gelatin solution, a definite constant product of $Ag_2CrO_4 = H$ exists which determines the limit of supersaturation for silver chromate in the absence of the solid phase."

Morse [1930] gives extensively the evidence in favour of occurrence of supersaturation and the rapid release of salt from a greatly supersaturated solution. Fricke [1923] writes:

"There is no reason for assuming a transient diminution of solubility during the formation of deposits. To do without the assumption of a more or less well-defined metastable limit in the sense of W. Ostwald, *i.e.* an ion limit product in order to explain the theory of Liesegang rings seems to be impossible."

Notboom [1923] also makes use of the idea of the metastable limit and in support quotes the development of this idea from consideration of surface energy as viewed by Haber and Vollmer.

It is in this sense of metastable limiting solubility that the term "solubility" is employed in this paper, and a limiting "solubility" product is considered as being applicable.

[1] When the formation of bands, as for example by CrO_4 entering into $AgNO_3$ in gel in a capillary tube under the influence of a superimposed E.M.F., is observed under the microscope, it is seen that the advancing CrO_4 colours the gel a faint orange, and behind the wave front of the CrO_4 a band appears as a sudden darkening of the gel increasing rapidly in intensity. By suitable choice of E.M.F. and concentration of reactants, bands can be produced at regular intervals of space and time (about 50 seconds or so).

"Solubility" product of Ag_2CrO_4. Approximate results were obtained by mixing standard solutions of $AgNO_3$ in 5 % gelatin and K_2CrO_4 in 5 % gelatin in equivalent quantities, allowing the gel to set and then causing $AgNO_3$, of different concentrations, to diffuse into the tubes, noting the tube which just gave faint precipitate at the surface (or, better, just a red coloration). Varying concentrations of entering $AgNO_3$ and of Ag_2CrO_4 in the gel all gave, for the same gelatin (5 % gel), a "solubility" product of the order of $(2{\cdot}2 \text{ to } 2{\cdot}9)10^{-7}$.

For 5 % ash-free gelatin the limiting "solubility" was between $N/166$ and $N/182$, giving a (mean) "solubility" product 1×10^{-7}. Bands of silver chromate can be obtained when concentrated $AgNO_3$ (*e.g.* N) diffuses into a gel containing Ag_2CrO_4 as weak as $N/1250$.

These results, rough as they are, indicate that the solubility product holds good for the "solubility" of Ag_2CrO_4 in gelatin.

The actual amount of Ag_2CrO_4 remaining in the clear spaces between bands must be less than the "solubility" measured as just described, *i.e.* lower than $0{\cdot}0085\ N$, because there must be subsequent separation of Ag_2CrO_4 due to the desaturating effect of that already separated. The reduction in solubility due to oncoming Ag, or to a less extent, to CrO_4, should not greatly affect the amount of total Ag_2CrO_4 actually found in the clear spaces, though it will precipitate some as solid particles in the clear spaces. This explains also why clear spaces formed in the early stages of diffusion subsequently fill (particularly noticed when rings are close together, as for example, when K_2CrO_4 is in the gel). The following are results showing the amount of Ag_2CrO_4 (calculated from the silver) in clear spaces—necessarily, to be wide enough for separate analyses, at some distance from the origin of the diffusion of the CrO_4 (or Cr_2O_7).

Table I.

(*a*) 0·1 *M* K_2CrO_4 diffusing into 0·01 *N* $AgNO_3$	(*b*) 0·2 *N* $K_2Cr_2O_7$ diffusing into 0·01 *N* $AgNO_3$
$[Ag_2CrO_4]$ in "clear" spaces	$[Ag_2CrO_4]$ in "clear" spaces
N	*N*
0·0076	0·0043
0·0068	0·0036
0·0061	0·0035
0·0061	0·0033
0·0046	0·0042

Obviously the concentration of the ion in the gel should not be very much greater than the limiting "solubility" if clearly spaced rings are required in the early stages of diffusion.

C. Development of the precipitate.

Once separation has occurred there is concentration of the precipitate by collection of the material from the intermediate zones. This is clearly seen if the formation of rings is followed by observation through the microscope, and particularly well if such observation is made when the rings are being produced under the influence of a superimposed E.M.F. An explanation of the mechanism of this concentration into more or less clearly defined bands is given by Jablczynski *et al.* [1923] who shows that it can be attributed to the growth of the relatively large particles at the expense of the smallest. This is a contributory effect in making bands more clearly defined; it is not the cause of band-formation. There is evidence in support of this theory in the behaviour of the primary rings which are often noticed when Ag_2CrO_4 forms in rings—particularly when $K_2Cr_2O_7$ is the internal reagent.

Where the main bands are rather close together, the primary bands show definite signs of being absorbed by the main bands. Where the main bands are relatively far apart, the central primary bands, being out of the "zone of attraction" into the main bands, may form a new "supplementary" band, and this probably explains the occurrence of bands in doublets and even triplets as frequently seen, in capillary tubes, where the rings are becoming more widely spaced, *i.e.* at some little distance from the surface where the electrolyte enters. Fig. 1, Plate V, showing the bands beyond the 18th band in a capillary tube, clearly suggests adsorption of the primary bands and indicates the formation of double bands.

D. Theory of formation of Liesegang rings.

(1) *Spacing of bands and its relation to the diffusion gradient of the entering salt (free ion).*

It has been found experimentally [Jablczynski, 1926] that the distances between bands are in the simple relationship

$$\frac{h_n}{h_{n-1}}=K, \quad \frac{h_n-h_{n-1}}{h_{n-1}-h_{n-2}}=K.$$

Morse [1930] states that "this indicates a ruling influence of diffusion and especially the influence of the diffusing (more concentrated) substance on ring formation and periodicity."

It is to be inferred that a constancy such as this depends on the cause(s) being of an unchanging nature. The diffusion of the entering ion fulfils this requirement; it proceeds so that the distance reached by the free ion is in accordance with $h/\sqrt{t}=K$. The corresponding requirement on the part of the ion in the gel is that its distribution, from the front of the last formed precipitate, throughout the unpenetrated part of the gel, shall remain reasonably constant. This is approximately true generally, but, as the ion contained in the gel moves forward to the entering ion, there must be some decrease in its concentration, with the consequence that the relation is not perfectly true over a long enough range, the change becoming noticeable in certain circumstances, such as when the concentration of the internal electrolyte is low and the entering ion has penetrated so far that its advance is very slow.

The effect on the spacing of the bands of variation in the concentration of the internal electrolyte is well illustrated by taking an exaggerated instance. For example, if a gradient of concentration of the internal electrolyte is produced by causing that electrolyte to diffuse into a gel, the other electrolyte which is subsequently caused to follow is thus diffusing into a gel containing electrolyte of gradually decreasing concentration (Fig. 2, Plate V); the second electrolyte may, instead, be introduced at the other end of the tube, thus travelling into electrolyte of increasing concentration.

A meaning can be found for the relationship as follows:

It is an experimental fact that the diffusion of the entering ion (or salt) X proceeds according to Fick's law whether or not any precipitating ion (salt) Y is contained in the gel. That is, although when precipitation does occur there is sudden separation of XY with temporary alteration of diffusing conditions, yet this does not amount to definite checking of the diffusion as the effect at any time is in accordance with the diffusion law (for a given external concentration C, $\frac{h}{\sqrt{t}}=K$). This is true so long as it is the "free" X which is considered.

In Fig. 3, CD represents the curve for "free" X when precipitation is just about to take place at A (h_{n-1}) where the concentration of X=metastable

"solubility," s. For a new precipitation to follow at B, (h_n), the concentration of "free" X at A, after precipitation at A, will have been made up to the original and have risen to an amount AF, so that CFE represents the "free" X curve when B is ready to precipitate, *i.e.* concentration at B has likewise reached the metastable "solubility," s.

Let it be assumed that when this value has been reached at B, the concentration of "free" X at A has reached a definite value, p, and that this is true for any ring when a new one is forming next to it.

For any given concentration of X along the tube, $\frac{h}{\sqrt{t}}=K$ for all positions of X of that given concentration: thus for concentrations of $X=s$, $\frac{h_{n-1}}{\sqrt{t_{n-1}}}=K_1$ and $\frac{h_n}{\sqrt{t_n}}=K_1$; for concentration $X=p$, $\frac{h_{n-1}}{\sqrt{t_n}}=K_2$.

$\frac{h_n}{h_{n-1}}$ then becomes $\frac{K_1}{K_2}=k=\left(\frac{\sqrt{t_n}}{\sqrt{t_{n-1}}}\right)$.

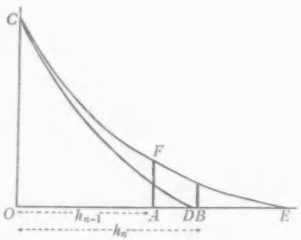


Fig. 3. CD and CE are curves for diffusion of the entering ion X.

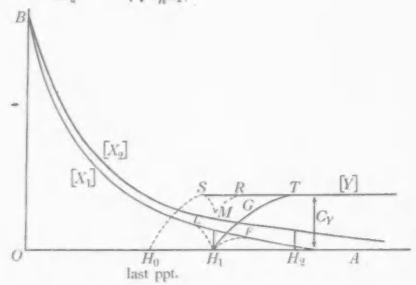


Fig. 4. Ordinate: Concentration (X and Y) Abscissa: Distance from surface.

So that, in order to satisfy the experimental facts that the ratio of distances of consecutive rings from the origin shall be constant, and that the times of formation shall be in accordance with the diffusion law, the requirements are:

(1) That ring-formation shall occur at a definite concentration of X (say $=s$). This follows from the fact that the concentration of Y, the other ion, is constant (uniformly distributed) so that, for the "solubility" product, which is constant, $[X]$ must reach a constant value.

(2) That when $[X]$ reaches this value (s), the concentration of "free" X at the ring previously formed must be of a certain constant value (p).

It is clear that the slope (or rather the flatness) of the diffusion curve governs the spacing of the bands, *e.g.*

M	K_2CrO_4 diffusing at 21° into 5 % gelatin, containing	$N/100$	$AgNO_3$	gave	$h/\sqrt{t}=1{\cdot}085$
$M/10$	,,	,,	,,	,,	$=1{\cdot}20$
$M/50$	,,	,,	,,	,,	$=1{\cdot}48$

The effect on the spacing, of the concentration of the contained salt (ion), is likewise to cause decrease for higher concentration, so that the position of the point B is governed by the gradient of the concentration of the contained ion at the precipitate front and it must be such that B (H_2 in Fig. 4) is under the horizontal position of that curve, *i.e.* beyond the actual gradient of concentration of the contained ion. This becomes evident in the following theoretical visualisation of the mechanism of band-separation, based on diffusion and the conception of metastable solution as proposed by several other workers, *e.g.* particularly Fricke [1923], Morse [1930] and others. The importance attached

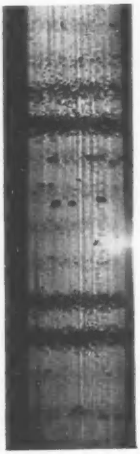
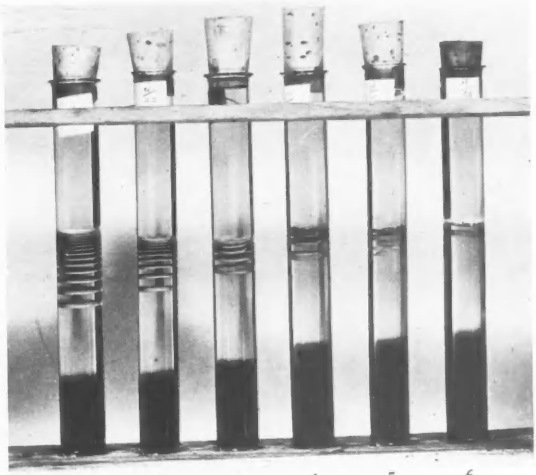


Fig. 1.

Fig. 2.



1 2 3 4 5 6

Fig. 7.

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to diffusion gradients, as here emphasised, has been realised solely from the analytical work recorded and is considered to afford definite confirmation of the theoretical considerations described by Fricke.

Theory of formation of Liesegang rings.

For simplification of description reference is made only to the ions which form the precipitate, and they are considered to be of equal valency. X is used to represent the entering "free" ion, and Y the ion contained in the gel. Suitable conditions of gradient of concentration of "free" X and concentration of Y are presumed (Fig. 4).

C_X = external concentration of X.
C_Y = concentration of Y (as originally uniform in the tube).
S = "solubility" (metastable) product.
S_X = concentration of X required to give "solubility" product S with C_Y.

Concentrations are amounts above final solubility of the product XY. Consider precipitation occurring at a point H_1. Here the metastable "solubility" product will have been reached, and, as C_Y is constant (approximately), S_X for ion X must be constant when precipitation occurs. X and Y now precipitate at H_1 and consequently there is a drop in concentration of the free ions, to the zero line (final solubility) for X, and to $C_Y - S_X$ for Y, as shown by the dotted lines at H_1, LH_1F [Fricke, 1923] and SMR respectively. As the amount of X, at H_1 and onwards, builds up again, so the Y in excess at H_1 and its neighbourhood is precipitated and collected into the precipitate at H_1, with the consequence that the concentration of Y at H_1 is gradually falling and that of X increasing again.

Finally the concentration of Y at H_1 is down to the final solubility of XY (the ultimate solubility of XY being that in presence of excess of X) and the conditions when precipitation again occurs at H_2 are represented for Y by the gradient from C_Y at H_2 to zero at H_1 (H_1GT) and, for X, by the curve X_2. The changes then re-occur as described for H_1 (though the whole procedure must be considered in general as continuous, except for the actual separation of XY at concentration of $X = S_X$). The amount of Y remaining between one precipitate and the next is precipitated ultimately, so that, to obtain clear spaces, it is essential that C_Y should be relatively only little higher than S_X.

Position of H_2 (B of Fig. 3).

It will be noted that, in the diagram, the positions of H_1 and H_2 have been shown as under the horizontal portion of the concentration curve for Y. The reason of this is as follows:

Referring again to the precipitation at H_2, Y moves along the gradient C_Y to zero at H_1 until interrupted by precipitation at H_2 with the consequence that the average (distribution) concentration of Y (as precipitate) is greater to the left of the last ring than to the right of it (experimental evidence, p. 1093). The concentration of free Y at H_1 is finally "zero." When precipitation takes place at H_2, this movement of Y to H_1 ceases, and so no further Y goes to the left. Consequently, rings once completely formed, except for the absorption of Y remaining in the last space or two, will no longer increase in amount of precipitate (experimental evidence, Graph 12). If H_2 were at a point in the actual gradient of Y to C_Y then the average (distribution) of Y finally remaining as precipitate would, for that distance, be less than C_Y, instead of slightly greater, a condition which does not occur. Consequently the zones of precipitation are

such that Y is at its standard concentration. The following analytical results, Table II, *a*, *b*, from some of the results previously given as curves, also illustrate this. In each group the results represent analyses made of identical tubes of gel after diffusion had proceeded for increasing periods of time, *e.g.* 24, 120, 172, 216 and 312 hours, and show the concentration of the internal ion in the zone of the precipitate and just beyond it, *i.e.* before the last formed ring has much intensified by collection from its neighbourhood. Such figures cannot, of course, be exactly representative of the newness or otherwise of a band, or exactly of the concentration in a band not very sharply defined, but they serve as an indication. In the precipitate, results for $[Y]$ greater than C_Y indicate that the precipitate had been formed for some time.

Table II.

a

0·5 *N* $AgNO_3$, *X*, into 0·04 *N* K_2CrO_4, *Y*

Front edge of precipitate	Portion of clear gel adjacent to precipitate
$[CrO_4]$ *N*	
0·039	0·020
0·044	0·020
0·044	0·010
0·047	0·012
0·043	0·011
0·042	0·012

b

0·1 *M* $K_2Cr_2O_7$, *X*, into 0·0075 *N* $AgNO_3$, *Y*

Front edge of precipitate	Portion of clear gel adjacent to precipitate
[Ag] *N*	
0·0097	0·0069
0·0095	0·0072
0·0105	0·0054
0·0083	0·0059

In view of the results and conclusions so far given, it becomes a simple matter to decide what conditions are necessary for the formation of banded precipitates or for continuous precipitate, presuming suitably slight solubility of the precipitate and the concentration of the contained ion above the metastable "solubility" of the precipitate.

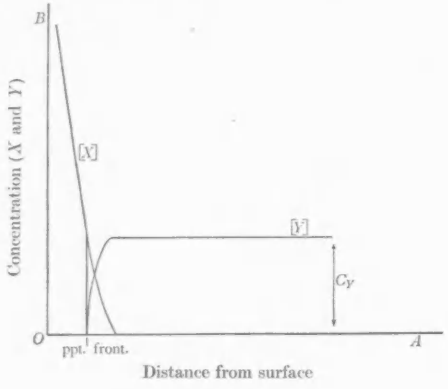

Fig. 5.

1. *Continuous precipitate* (Fig. 5).

The gradient of $[X]$ must be steep so that a slight movement along OA brings the requisite amount of X required for precipitation under C_Y. Likewise the gradient C_Y must be so steep that $[X]$, in an extremely small distance from

the precipitate front, comes to "solubility" concentration under the horizontal part of $[Y]$. This necessitates relatively high concentration of Y and much higher concentration of X. Unless C_X is really high compared with C_Y some band-formation will occur after diffusion has proceeded for some time and the gradient of $[X]$ has consequently become much less steep.

2. *Banded precipitate* (Fig. 6).

For the formation of widely spaced clearly separated bands, the gradient of $[Y]$ must be relatively flat, so that the next occurrence of the metastable limit, which will be for a position of the requisite amount of X under the horizontal portion of the $[Y]$ curve, will be some distance away from the last precipitate. The clearness of the spaces will depend on the amount of Y left between a new band and the previous one, and consequently the concentration of Y $[C_Y]$ should be low, this depending on the effect of X in reducing solubility of XY

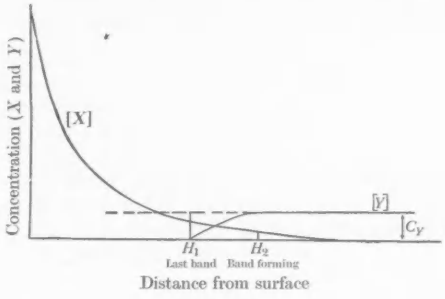

Fig. 6.

and also on the way in which XY can be absorbed into a band. The spacing will depend both on the concentration of Y, which must be low, and on the external concentration of X which also must be low (though, of course, higher than that of Y) in order that the gradient of $[X]$ shall be flat (as H_1 to H_2, Fig. 6).

As already explained, the spacing can be considered in terms of the diffusion of X only with allowance for the effect of $[C_Y]$ on it; *i.e.* as a balanced continuous process.

With conditions between those for continuous precipitate and for widely spaced clearly separated bands, any intermediate type of banding may be procured, *e.g.* rather close bands with fairly clear spaces; close bands with "spaces" filled with precipitate, the bands being only distinct as more intensely coloured and more compact; and so on.

When the gradient of $[X]$ is relatively steep and $[C_Y]$ is rather low, there will be a tendency for some continuous precipitate to form before the gradient of $[X]$ is flat enough to give banding. This is a matter of common observation.

If the $[X]$ gradient commences rather flat, it is possible that banding will commence at once. For example, Fig. 7, Plate V (1–6) shows the results of diffusion of $N/10$, $N/20$, $N/30$, $N/40$, $N/50$, $N/60$, K_2CrO_4 respectively into $N/100$ $AgNO_3$ in 5 % gelatin (for 64 hours).

Thus it is possible, allowing for conditions as described, to predict the character of band-formation and to adjust conditions accordingly.

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CXLIX. STUDIES IN ZYMASIS.

VII. THE ESTIMATION OF THE ETHYL ALCOHOL AND ACETALDEHYDE CONTENT OF APPLES.

By JOHN CARTER FIDLER.

From the Botany Department, Armstrong College, Newcastle-on-Tyne.

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The following paper presents an account of the methods used in arriving at the alcohol numbers [Fidler, 1933, 1] given in the former papers of this series [Fidler, 1933, 1, 2; Thomas and Fidler, 1933].

Suggestive information has been gained by previous workers (see literature quoted by Kostytschew [1927] and Thomas [1925]) as to the progress of alcohol production by methods that had not been standardised. Little value, however, can be attached to numbers purporting to represent relations between alcohol and carbon dioxide production. Moreover, we consider that when anaerobic respiration forms one component of the total respiration it is essential to know the accuracy with which the various zymasic products and the respirable substrates have been measured, before any attempt is made to construct a balance sheet.

The results of this critical examination of a specialised analytical technique are presented in summary tables in this paper, and it will be seen that in all cases the standard deviations of the results are known. In view of the detailed discussion of the technique that was to be given in this paper, no mention of the accuracy of the methods was made in the earlier papers of this series, but only significant figures were given in the tables. It will now be seen that we are able to determine whether any experimental treatment can be said significantly to have increased the alcohol number of an apple.

The relation between the actual alcohol number of an apple at the end of an experimental treatment to the production of ethyl alcohol and acetaldehyde during the period of the experiment is discussed in appendices.

I. Estimation of ethyl alcohol and acetaldehyde in physical systems.

(The results of the different sub-sections are given in the summary Table I.)

A. In aqueous solutions.

1. *Acetaldehyde.*

(*a*) *By the method of Ripper* [*1900*]. Ripper's method is so well known that the details need not be given here. It has been tested, using a pure specimen of acetaldehyde, purchased from Kahlbaum, and found to be accurate to within the limit given by Ripper, namely $\pm 0{\cdot}3$ %. In four trials of the method, using

different volumes of an acetaldehyde solution made up from a weighed quantity of the pure liquid, the correspondence between the weighed quantity of acetaldehyde and the quantity determined by the fixation with sodium hydrogen sulphite was within 0·1 mg. in quantities ranging from 19·7 mg. to 39·4 mg. of acetaldehyde.

It may be thought that an error would be introduced into the acetaldehyde figure found by this method, due to autoxidation of the sodium hydrogen sulphite. However, using $N/20$ iodine solution in the titrations, no oxidation of the bisulphite solution could be detected in an hour, the liquid standing with a little air in a corked flask in melting ice.

(*b*) *By oxidation with potassium dichromate.* It has been found that acetaldehyde may be determined by oxidation with potassium dichromate in acid solution, the resulting volatile acid being then distilled off and estimated by titration with standard NaOH solution.

A series of oxidations was carried out with amounts of acetaldehyde ranging from 0·8 to 78·7 mg. under standard conditions, the concentration of sulphuric acid being maintained at 2·5 % by volume and that of potassium dichromate at 5 % by weight.

Aqueous solutions of acetaldehyde of approximately the strengths required were standardised by Ripper's method, and 10 ml. were added to each of three bottles containing 10 g. pure potassium dichromate and 5 ml. pure sulphuric acid, dissolved in 185 ml. of distilled water. The bottles were closed by ground glass stoppers and allowed to stand 18 hours in melting ice, after which the contents were transferred to distilling flasks. The liquids were distilled down to about 50 ml., and water was then added to the contents of the flask. These distillations and additions of water were continued as long as any volatile acid distilled over. Each 500 ml. of distillate was titrated with $N/10$ CO_2-free NaOH, using phenolphthalein as an indicator, and the distillations were continued until 500 ml. of the distillate required an amount of NaOH equivalent to that required by a distillation of a mixture of 10 g. potassium dichromate, 5 ml. sulphuric acid and 200 ml. of water.

This last titration reading is not included in the calculation of the total acidity of the distillates. From the total amount of NaOH required, the acetaldehyde content of the original solution was calculated, on the basis of the equations:

$$2CH_3.CHO + O_2 \rightarrow 2CH_3.COOH,$$

and

$$CH_3.COOH + NaOH \rightarrow CH_3.COONa + H_2O$$

and in every case was found to be greater than that found by Ripper's method. Since we may rely on the accuracy of Ripper's method, it is clear that some acidic substance other than acetic acid is produced by the oxidation of acetaldehyde by potassium dichromate in acid solution[1].

Experiments carried out in triplicate in ten different concentrations within the range indicated above gave a mean percentage excess of acetaldehyde by the oxidation method, as compared with Ripper's method, of 10·1 %, with a standard deviation of 2·51. The results are plotted in Fig. 1, where a line has been drawn through the origin, making an angle of 47° 45′ with the x-axis. The

[1] Part of the distillate, carefully neutralised with CO_2-free NaOH, did not induce turbidity in baryta solution containing barium chloride, so that the excess acidity is not due to SO_2 or CO_2. It may be due to formic acid. Conant and Tongberg [1930] have shown that the normal course of the oxidation of acetaldehyde with ceric sulphate and sulphuric acid is to formic acid, and claim to have demonstrated α-oxidation of acetaldehyde in acid potassium dichromate solution.

tangent of this angle is 1·101. This means that the values of the abscissae exceed those of the ordinates by 10·1 %. This line passes through or very near to the points of the figure. The graph further shows that the deviation from the mean is of the order of 0·5 mg. in the lower concentrations, and never greater than 2·0 mg. in the high concentrations. The sections dealing with estimations of mixtures of ethyl alcohol and acetaldehyde will illustrate the value of the above oxidation method (*e.g.* see III A).

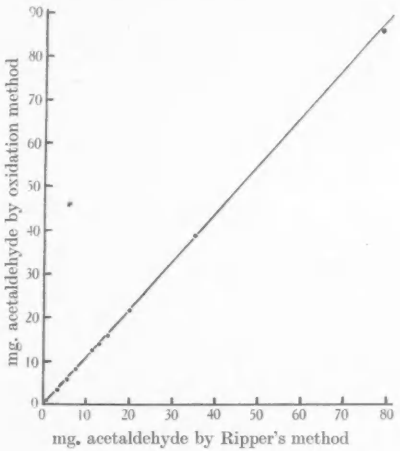

Fig. 1.

Further experiments, in which the concentration of the sulphuric acid was increased to 5 and 15 % and the time of oxidation to 3 days at 15°, gave figures which did not deviate from the mean value of 10·1 % excess by more than 2 × S.D., so we may conclude that the conditions of the oxidation may be allowed to vary within limits. However, all oxidations of acetaldehyde described in this paper have been carried out in 2·5 % sulphuric acid for 18 hours at 0°.

2. *Ethyl alcohol.*

(*a*) *By oxidation with potassium dichromate.* Dox and Lamb [1916] describe a method[1] of estimating small quantities of ethyl alcohol by oxidation to acetic acid and titration of this compound with standard alkali. The method has been tested over a range of alcohol concentrations, and the results are given in Table I. The alcohol added was estimated by determination of the sp. gr. in a pyknometer, and reference to tables. All oxidations were carried out in 500 ml. solution, containing 12·5 ml. concentrated sulphuric acid and 10 g. potassium dichromate. The oxidation mixture was allowed to stand for half an hour at room temperature and then distilled, with repeated additions of water to the contents of the flask. The distillates were titrated against $N/10$ CO_2-free NaOH until the figure required to neutralise the distillate from the distillation of a mixture of sulphuric acid, potassium dichromate and water was reached. The first titration reading equal to this is included in the calculation of acidity.

[1] First described by Dupré [1867] and given in Allen [1901].

Table I. *Recovery of acetaldehyde and ethyl alcohol from physical systems.*

Substance added	Method of: Standardisation of solution	Method of: Estimation of recovery	No. of different weights of substances added	Total no. of observations	Range of weights of substances added (g.): From	Range of weights of substances added (g.): To	Mean percentage recovery	Deviations from mean percentage recovery: In lowest conc. +	In lowest conc. −	In highest conc. +	In highest conc. −	Maximum observed +	Maximum observed −	Standard deviation (S.D.)
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
(1) Estimation in aqueous solution:														
Acetaldehyde	Weighed amount of acetaldehyde in definite volume of water	Ripper	2	4	0·0197	0·0394	100·25	Nil	Nil	Nil	Nil	Nil	Nil	Nil
„	Ripper	Oxidation	10	10	0·0008	0·0787	110·1 (*a*)	3·0	—	—	1·0	3·0	6·0	2·51
Ethyl alcohol	Pyknometer	„	7	16	0·0421	0·2185	100·1	1·1	1·3	0·4	0·8	1·2	1·3	0·99
(2) Following steam-distillation of an aqueous solution:														
Acetaldehyde	Ripper	Ripper	6	15	0·00120	0·02126	98·0	5·2	1·2	—	0·9	5·2	3·1	1·97
„	„	Half distillate oxidised. Other half: Ripper	2	8	0·0292	0·0600	97·0 (*b*) 98·3	3·0 1·7	5·5 1·1	2·2 1·7	0·2 2·6	3·0 1·7	5·5 2·6	2·60 1·54
Ethyl alcohol	Pyknometer	Oxidation	7	17	0·0466	0·1718	100·2	1·4	2·3	—	1·5	1·4	2·3	1·09
Acetaldehyde	Ripper	Ripper	6	24	0·0058	0·0140	98·8	—	2·1	1·9	1·7	2·8	3·3	1·74
Ethyl alcohol	Pyknometer	Difference	2	24	0·0467	0·1167	100·4	3·0	1·8	0·4	1·3	3·0	1·8	1·03
Alcohol number	Sum	Oxidation	6+2 (*c*)	6+24	0·0525	0·1307	100·2	2·4	1·9	1·2	0·9	2·4	1·9	0·97

(*a*) Recovery calculated on assumption that all volatile acid is acetic acid.
(*b*) This figure cannot be compared with (*a*) since these recoveries were calculated by assuming that the acetaldehyde equivalent of the volatile acid was 100/110·1 (see Fig. 1).
(*c*) This series includes six experiments in which the recovery of acetaldehyde from the mixture was not estimated, but assumed to be 100 %.

Table I shows that the mean percentage recovery in sixteen trials of the above method, with amounts of ethyl alcohol ranging from 0·0421 to 0·2185 g., was 100·1, and the S.D. of the method was 0·99. This is a high order of accuracy for an organic analysis, and the method is ideally suited to the estimation of ethyl alcohol in low concentrations in aqueous solution.

B. In distillates obtained from steam-distillation of aqueous solutions.

It is pertinent to the present enquiry to know whether distillation in a current of steam and condensation of the distillates lead to any loss of ethyl alcohol, or acetaldehyde, from aqueous solutions containing these substances, either singly or in mixtures with each other. Details concerning the solutions which have been distilled are given in Table I. All solutions were distilled for 2 hours, the distillates being collected as described in Section III A.

1. *Acetaldehyde.*

It has been found impossible to avoid loss of acetaldehyde in steam-distillation, while taking the greatest care to guard against losses. When the acetaldehyde content of the distillate was determined by Ripper's method, the average recovery in fifteen trials, in which the weight of acetaldehyde ranged between 0·0012 and 0·0213 g., was 98 %, with a S.D. of 1·97. Using the oxidation method of estimation, the average recovery fell to 97 %, and the S.D. rose to 2·6. This error is of the same order as that introduced by the oxidation. So we conclude that, on the average, 3 % of this very reactive substance has been lost, possibly by undergoing some chemical change at the high temperature. It is possible for the loss to be as high as 8 % in extreme cases, although the maximum observed loss was 5·5 %.

2. *Ethyl alcohol.*

Seventeen trials of the effect of steam on ethyl alcohol solutions were carried out. In these the weights of ethyl alcohol varied from 0·0466 to 0·1718 g. The average percentage recovery was unaffected by the steam-distillation treatment, and the errors of estimation appeared to be of the same order as those recorded under A (2).

3. *Mixtures of ethyl alcohol and acetaldehyde.*

Mixtures of standard solutions of these substances in different proportions were distilled and the distillates worked up by the standard method described in Section III A. We now encounter for the first time in this discussion variations in the estimates of the "alcohol number." The standard method, it will be seen later, gives a direct measure of the acetaldehyde content and the alcohol number, but the ethyl alcohol concentration is given by the difference between these figures and should therefore be subject to a variation which is a function of the separate variances of the acetaldehyde and alcohol number figures. It is therefore all the more gratifying to find that the mean recovery of ethyl alcohol, as found by difference, was of the same order as that in the modified Dox and Lamb estimation (A (2)) and the standard deviations of the two sets of figures were essentially the same.

The figures in Table I provide evidence that the presence of ethyl alcohol does not affect the recovery of acetaldehyde in a steam-distillation.

II. Estimation of ethyl alcohol and acetaldehyde in biological systems.

Since it was uncertain whether ethyl alcohol and acetaldehyde could be recovered quantitatively from biological material by a steam-distillation technique, a series of experiments was carried out on the recovery of known quantities of these substances from sliced apple tissue.

The sliced tissue (York Imperial, Newton Wonder or Bramley's Seedling apples) was well washed and mixed, and to separate 100 g. samples were added definite volumes of standard solutions of ethyl alcohol or acetaldehyde. The results are given in summary in Table II, and are discussed in some detail in the subsections below. No appreciable differences could be detected between the basal ethyl alcohol or acetaldehyde contents of separate 100 g. samples of the mixed tissue, using the analytical methods described under III A. The tissue was freshly prepared for each series of experiments. Usually four tests were made of any given concentration of the substance studied. All the mixtures stood half an hour at room temperature in a closed distilling flask before the current of steam was started. A distillation time of 2 hours was taken as the standard, and the figures in column 9 of Table II indicate that such a period is a satisfactory one.

The knowledge obtained from the results of the experiments described in I B is very necessary, because at the present time it seems that steam-distillation appears to be the best method of recovering ethyl alcohol and acetaldehyde from plant tissues, and especially from apples, in a short enough space of time to preclude secondary changes.

(A) Acetaldehyde and ethyl alcohol supplied singly.

A comparison of the mean percentage recoveries of either of these two substances in purely physical systems and in the presence of apple tissue (Tables I and II) shows that there are no striking differences between the two sets of figures. (In the case of ethyl alcohol the two means are identical.) There appears, however, to be more swing about the mean in the figures obtained from the biological systems. Thus, in the case of the recovery of acetaldehyde, the estimated S.D. of the mean has risen from 1·97 to 2·44, and similarly for ethyl alcohol from 1·09 to 1·56. Thus the maximum deviation to be expected, as a pure matter of chance, once in every twenty estimations, has risen from 4 to 5 % for acetaldehyde, and from 2 to 3 % for ethyl alcohol.

(B) Mixtures of acetaldehyde and ethyl alcohol.

The alcohol number. In estimations of products of zymasis in fruits, where the size of the sample is so large that sampling errors can be neglected (a state of affairs rarely realised), the results of this subsection will be the most important.

We will consider at this stage only the estimates of the S.D. given in column 16 of Table II. These estimates have been calculated by the same formula as used in the earlier subsections and are comparable with those given in Table I. Although the mean recovery of either acetaldehyde or of ethyl alcohol is not altered by the presence of the apple tissue during the distillation, there is greater deviation from this mean value. Thus the estimated S.D. has risen in each instance. Our published results deal with the alcohol numbers of apples,

Table II. *Recovery of acetaldehyde and ethyl alcohol from biological systems.*

Substance added	Method of: Standardisation of solution	Method of: Estimation of recovery	Variety of apple	No. of different weights of substances added	Total no. of observations	Range of sum of weights of substance added + amount present in tissue (*a*): From	Range of sum of weights of substance added + amount present in tissue (*a*): To	Mean percentage recovery	Deviations from mean percentage recovery: In lowest conc. +	In lowest conc. −	In highest conc. +	In highest conc. −	Maximum observed +	Maximum observed −	Standard deviation (s.d.): Method 1 (*b*)	Standard deviation (s.d.): Method 2 (*c*)
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Acetaldehyde	Ripper	Ripper	York Imperial	4	4	0·0092	0·0305	95·2	4·8	—	0·8	—	4·8	4·3	3·75	—
			Newton Wonder	6	24	0·0025	0·0241	97·8	8·2	—	0·5	0·7	8·2	3·8	2·64	—
			Bramley's Seedling	6	24	0·0038	0·0254	98·1	3·5	—	—	0·5	3·5	2·0	1·62	—
			All three varieties	16	52	0·0025	0·0305	97·7	8·3	—	—	3·4	8·3	6·8	2·44	—
Ethyl alcohol	Pyknometer	Oxidation	York Imperial	6	6	0·0574	0·4257	99·4	0·6	—	—	1·0	1·4	1·0	0·68	—
			Newton Wonder	4	16	0·0258	0·1728	100·1	2·6	3·6	0·2	0·9	2·6	3·6	1·65	—
			Bramley's Seedling	5	20	0·0252	0·2396	100·6	1·3	—	2·7	7·5	2·7	7·5	1·95	—
			All three varieties	15	42	0·0252	0·4257	100·2	1·7	—	0·5	1·9	3·1	6·1	1·56	—
Acetaldehyde	Ripper	Ripper	Newton Wonder	6	24	0·0053	0·0212	99·6	0·4	5·3	2·9	—	3·8	3·0	2·12	1·77
			Bramley's Seedling	4	24	0·0054	0·0204	97·8	0·4	3·2	4·2	0·3	4·2	5·2	2·45	2·05
			Both varieties	10	48	0·0054	0·0212	98·7	1·3	4·4	2·0	0·8	4·7	4·4	2·28	1·92
Ethyl alcohol	Pyknometer	Difference	Newton Wonder	2	24	0·0255	0·0885	100	5·5	7·0	2·6	2·8	5·5	7·0	3·68	4·16
			Bramley's Seedling	2	24	0·0261	0·0986	100·5	4·9	5·5	2·7	4·5	4·9	5·5	2·86	3·16
			Both varieties	4	48	0·0255	0·0986	100·2	7·5	7·2	2·9	4·2	7·5	7·2	3·33	3·69
Alcohol number	Sum	Oxidation	Newton Wonder	6	24	0·0308	0·1097	99·7	6·1	—	0·3	2·2	6·1	3·4	2·38	1·53
			Bramley's Seedling	6	24	0·0315	0·1140	100	3·2	1·6	1·8	3·7	3·2	3·7	2·36	2·38
			Both varieties	12	48	0·0308	0·1140	99·8	6·0	—	2·0	3·5	6·0	3·6	2·33	2·00

(*a*) The basal values for acetaldehyde and ethyl alcohol contents of the tissues were determined by the method described in Section III. They are subject to errors of estimation, but it would be an unnecessary refinement to allow for these errors.

(*b*) Throughout Sections I and II, the actual ethyl alcohol and acetaldehyde contents of the tissues were known, and hence the recoveries of these substances could be expressed as percentage recoveries. Considering any separate series from either of these two Sections (*e.g.* the series of trials of the recovery of ethyl alcohol from Bramley's Seedling apple tissue) the s.d. is found by the formula $\text{s.d.} = \sqrt{\frac{\Sigma (x-\bar{x})^2}{n-1}}$ where $\Sigma (x-\bar{x})^2$ = the sum of the squares of the differences between each percentage recovery (x) and the mean percentage recovery of the series ($\bar{x}$), and n = the total number of experiments in the series. Thus $n-1$ gives the number of degrees of freedom. Only column 16 will be considered in this section.

(*c*) The method of calculation of column 17, and further reference to this column, is deferred until Section III, in the notes to Table III.

and, if perfectly even sampling is assumed, it will be seen from the S.D. of the alcohol number that a deviation of 4·66 % from the true value is to be expected once in every twenty estimations as a matter of chance.

Up to the present, our studies have not necessitated an accurate knowledge of the ethyl alcohol content of tissues (as distinct from the alcohol numbers), but were we to calculate these figures from the percentage figures for acetaldehyde and the alcohol number, we should expect the odds against any figure deviating by as much as 7 % from the true value to be 19 : 1 (again neglecting sampling error).

III. Variations between small samples.

The data given in the previous section are of use in demonstrating that the projected method is applicable to the assay of alcohol numbers of apples. The figures are of little use, however, in analysis of actual experimental results obtained with whole apples, since the errors introduced by random sampling greatly exceed the errors in the analytical technique. Estimates of total errors (which include sampling errors) for samples of two apples each have been obtained for two varieties of apples. The experiments described in Nos. IV, V and VI of this series [Fidler, 1933, 1, 2; Thomas and Fidler, 1933] were done with samples ranging from one to three mature fruits, for reasons given in the sixth paper. Thus the samples for the experiments described in this section were of two apples each. As will be seen later, it is easy to calculate the error for a sample of any other size.

The results are given in Table III, and are discussed in Subsection B. Acetaldehyde and ethyl alcohol were formed in the apples by exposing them to the experimental conditions summarised in Table III, in the apparatus described by Fidler [1933, 1], for definite lengths of time. The experiments were arranged in groups, each of which comprised three comparable samples of two apples each. At the end of the experimental treatment, the separate samples of each group were distilled and the acetaldehyde and ethyl alcohol contents of the distillates determined in the manner described in Subsection A.

The figures given in Table III for ethyl alcohol and the alcohol number include the transpired alcohol, as did the figures in the earlier papers. This transpired alcohol is determined as described in Appendix II.

The apples were from the population stored for our use by Dr Cyril West, at the Ditton Laboratory, East Malling, Kent, during the season 1932–3.

A. Apparatus and method.

After removal of the apples from the experimental conditions, the alcohol and acetaldehyde were extracted by steam-distillation in the apparatus first used and described by Thomas [1925].

Passing through the stopper of a 2-litre flask, containing 1500 ml. water, is the narrow stem of a glass cylinder, 30 in. by 2½ in., the top of which is in communication by means of a splash trap with a 10 in. water condenser of the double surface type. The cylinder is well lagged with cotton-wool. The condenser is in turn connected to a tube passing through the stopper of a 1-litre flask to within ¼ in. of the bottom. This flask is connected to a second similar receiver. Each receiver contains 50 ml. distilled water and stands in a trough filled with melting ice.

Before use, the apparatus is disconnected at the top of the glass cylinder, and the water in the flask is boiled until the issuing steam has heated the walls of the cylinder. The apples are now cut, each into eight pieces, and dropped into the cylinder, where they are retained by a circle of

perforated zinc, the edges of which are bound with rubber. The apparatus is immediately reconnected and distillation proceeds for 2 hours.

When the distillation is completed, the distillates are combined and made up to 1 litre with water. Half of this liquor is used for the determination of acetaldehyde by Ripper's method[1] and the other half is oxidised by the addition of a solution of 15 ml. concentrated sulphuric acid and 25 g. potassium dichromate in 85 ml. water. The oxidation is allowed to proceed for 18 hours in ice. The conditions may be allowed to vary a little, as indicated in Section I A 1 (*a*), which also describes the method of distillation of the oxidation-liquors. It is advantageous to use Claisen flasks for these last distillations, and it is important that no traces of colour should be present in the acid distillates. The liquid in the flasks should be distilled to small bulk (about 50–60 ml.) after each addition of water. In this way it is found that three additions and evaporations of water will suffice to carry over very large amounts of volatile acid.

The calculations of the ethyl alcohol content, and the alcohol number are carried out in the following manner.

Let A = ethyl alcohol content of the distillate.

Let B = acetaldehyde content of the distillate (by Ripper's method).

Then the ethyl alcohol equivalent of $B = \frac{44}{46} B$.

On oxidation, B will yield volatile acid equivalent to $\frac{11}{10}^{*} \times \frac{44}{46} B$, expressed as ethyl alcohol.

C, the total ethyl alcohol equivalent of the distillate from the oxidation

$$= A + \left(\frac{11}{10} \times \frac{44}{46} B\right) \text{ or, } A = C - 1{\cdot}05\, B.$$

The alcohol number $= A + \frac{44}{46} B = A + 0{\cdot}96\, B$.

Of the two alternative methods of approach to the problem of estimation of ethyl alcohol in the presence of acetaldehyde, namely (1) removal of the acetaldehyde as the initial step in the analysis, and (2) oxidation of the whole solution after estimation of the acetaldehyde fraction by Ripper's method, the second has not previously been used. Despite numerous criticisms, it has been the universal practice of workers studying the course of fermentation to follow the first procedure. The acetaldehyde has been removed by, *e.g.* sodium hydrogen sulphite [Kostytschew and Hübbenet, 1912], or *m*-phenylenediamine hydrochloride [Neuberg and Hirsch, 1919]. The solutions are boiled under a reflux condenser to ensure condensation of the reagent with acetaldehyde, neutralised and evaporated at reduced pressure to remove the ethyl alcohol, which is condensed, or collected in sulphuric acid [Thomas, 1925]. The latter is probably the better practice.

Tomoda [1929] reviewing these, and related methods, describes them as "tedious and troublesome in manipulation" and states that loss of ethyl alcohol or incomplete removal of acetaldehyde may readily occur.

[1] *Estimations of acetaldehyde in the presence of HCN and H_2S.* As yet unpublished work by Thomas in 1929–31 and Thomas and Fidler during 1931–3 on the effect of HCN and H_2S on zymasis by apples necessitated slight modifications of Ripper's method. The blue end-point with starch and iodine is impermanent in the presence of HCN, and practice is needed in order to judge the correct end-point. With care, however, this may readily be done. The question is more involved, however, in the presence of H_2S, for iodine is reduced by H_2S. Our procedure when the distillates have contained this substance has been to oxidise one-third only, using one-third for the estimation of H_2S by titration with iodine. An amount of $N/20$ iodine equivalent to that used in this titration was then added to the third portion of the distillate, oxidising the H_2S. H_2S thus being removed, the estimation of acetaldehyde proceeded in the normal manner.

* From Fig. 1.

Table III. *Errors in estimations with samples of two apples each.*

Variety of apple	No. of exp. (in order of conc. of ethyl alc.)	Date exp. begins	Initial weight of sample (g.)	% oxygen in atmosphere of exp.	State of apples	Duration of exp. (hrs.)	g. per 100 g. apple per 100 hrs. Acetaldehyde	g. per 100 g. apple per 100 hrs. Ethyl alc. (allowing for transpired alc.)	% alc. no.	Increase in % alc. no. in 100 hrs. exposure to gas	% CO_2 production (g./100 hrs.)	CO_2 : alcohol no. (both expressed as gram-molecule equivalents)	s.d. (*a*) Acetaldehyde %	s.d. (*a*) Alc. no. or ethyl alc. %	s.d. (*a*) CO_2 %	s.d. (*a*) CO_2 : alc. no. %	2 √2 (s.d.) (*b*) Acetaldehyde %	2 √2 (s.d.) (*b*) Alc. no. or ethyl alc. %	2 √2 (s.d.) (*b*) CO_2 %	2 √2 (s.d.) (*b*) CO_2 : alc. no. %
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Newton Wonder	1 *a*	17. xi. 32	275·0	7·0	Green	96·0	0·0003	0·0117	0·0120	—	0·2389	—								
	b	,,	290·0	,,	,,	,,	0·0003	0·0091	0·0094	—	0·2461	—								
	c	,,	279·0	,,	,,	,,	0·0003	0·0117	0·0120	—	0·2475	—								
	2 *a*	26. x. 32	225·0	5·0	Green	72·0	0·0011	0·0137	0·0148	—	0·2315	—								
	b	,,	232·0			,,	0·0011	0·0138	0·0149	—	0·2169	—								
	c	,,	235·0	,,	,,	,,	0·0008	0·0134	0·0142	—	0·2137	—								
	3 *a*	19. x. 32	260·0	Nil	Green	119·0	0·0013	0·1521	0·1534	0·1500	0·2538	100:56·5								
	b	,,	306·0	,,	,,	,,	0·0014	0·1764	0·1778	0·1778	0·2760	100:60·4	13·9	8·7	5·0	6·2	40	25	14	18
	c	,,	296·0	,,	,,	,,	0·0017	0·2009	0·2026	0·1992	0·3160	100:65·9								
	4 *a*	7. ii. 33	222·5	3·5	Yellow	71·5	0·0027	0·2177	0·2204	—	0·3077	—								
	b	,,	213·0	,,	First sign of wrinkling	,,	0·0035	0·2290	0·2325	—	0·3164	—								
	c	,,	217·5	,,	,,	,,	0·0027	0·2210	0·2237	—	0·3080	—								
	5 *a*	10. xi. 32	226·5	Nil	Green	95·5	0·0042	0·2442	0·2485	0·2430	0·3430	100:67·8								
	b	,,	226·0	,,	,,	,,	0·0036	0·2455	0·2485	0·2428	0·3153	100:73·6								
	c	,,	235·0	,,	,,	,,	0·0033	0·2418	0·2451	0·2394	0·3294	100:69·5								
Bramley's Seedling	6 *a*	16. xii. 32	256·0	21·0	Green to yellow-green	96·0	0·0030	0·0611	0·0641	—	0·4033	—								
	b	,,	240·5	,,		,,	0·0026	0·0566	0·0592	—	0·3922	—								
	c	,,	256·0	,,	,,	,,	0·0026	0·0620	0·0645	—	0·3748	—								
	7 *a*	1. xii. 32	261·5	7·6 then in air 10 days at 15°	Green	48·5 + 240	0·0047	0·0998	0·1045	—	0·3280	—								
	b	,,	281·5		,,	,,	0·0045	0·0920	0·0965	—	0·2638	—								
	c	,,	250·0		,,	,,	0·0046	0·0964	0·1010	—	0·2927	—								
	8 *a*	1. ii. 33	223·5	3·5	Yellow-green	95·0	0·0031	0·1376	0·1407	—	0·2828	—								
	b	,,	228·5	,,	,,	,,	0·0027	0·1151	0·1178	—	0·2444	—								
	c	,,	222·5	,,	,,	,,	0·0026	0·1234	0·1260	—	0·2601	—								
	9 *a*	24. xi. 32	260·5	Nil	Green	95·5	0·0040	0·3633	0·3673	0·3632	0·4085	100:85·0								
	b	,,	265·0	,,	,,	,,	0·0030	0·3269	0·3299	0·3258	0·3625	100:86·0								
	c	,,	262·5	,,	,,	,,	0·0034	0·3649	0·3683	0·3642	0·3958	100:92·1								
												Considering both varieties together ...	12·1	7·7	6·2	5·6	34	22	18	16

(*a*) *The* S.D. In the experiments described in this section where the variation between samples of two apples, considered in groups of three samples, is studied, the actual contents of acetaldehyde and ethyl alcohol were unknown. Thus deviations can only be expressed, within each group, as deviations from the mean value for the group. The formula used in Sections I and II (see note (*b*) to Table II) for estimating the S.D. cannot be used in this case. We have instead to determine the square of the difference between each individual value within a group and the mean value of the group, and to sum the resulting figures for the whole series. We thus obtain an expression: $\Sigma d^2 = \Sigma (x - \bar{x}_1)^2 + \Sigma (x - \bar{x}_2)^2 \ldots + \Sigma (x - \bar{x}_m)^2$, where x is any individual value and $\bar{x}_1$, $\bar{x}_2$, and $\bar{x}_m$ are the different mean values for the different groups. The S.D. is given by $\sqrt{\frac{\Sigma d^2}{n-m}}$ where n = total number of experiments, m = total number of groups of experiments, and $n-m$ = number of degrees of freedom.

It is useful to know how far any result may differ from the mean value of the population, and 2 (S.D.) will give the percentage deviation from the mean within which 95 % of the observations will lie, as a matter of chance.

(*b*) It is often useful in biological work to know the probability of two independent observations differing by a given amount. The odds are 19 to 1 against any two random samples from the same population of apples differing by more than the percentage values given in this column. The well-known expression for this is 2 √2 (S.D.).

(*c*) All experiments were carried out at 23°.

The alternative procedure of oxidising the whole solution has the merit of being simple in manipulation, involving only those operations which would be necessary in estimating the alcohol content of a solution were it first of all freed from acetaldehyde. Little apparatus is necessary and, since the oxidation proceeds overnight, no time is lost.

B. Results.

The results of the experiments on Newton Wonder and Bramley's Seedling apples are given in Table III.

When the estimates of S.D. given in Table III are compared with those given in column 17 of Table II, it is immediately evident that random sampling has introduced an error greatly in excess of that due to the actual estimations.

From Table III it is seen that there must be very great differences between the values for any zymasic product in different samples of two apples each, before it can be said with certainty that the values are significantly different. Thus *e.g.* the alcohol numbers of two samples, each of two Bramley's Seedling apples, from different experimental conditions, must differ by more than 23 % before we are certain that the different conditions of the experiments have resulted in different values for the alcohol numbers.

Fortunately, there exists a well-known method for reducing the error introduced by random sampling. If the S.D. of a single observation is known, then the S.D. of the mean of n observations from comparable samples is $\frac{\text{S.D.}}{\sqrt{n}}$. This formula may be used in another way. Thus the S.D. of a sample of N apples, when the S.D. of a sample of size n is known is given by $\text{S.D.}_N = \frac{\text{S.D.}_n}{\sqrt{N/n}}$. If *e.g.* the sample size is increased to 20 apples, the S.D. of a single observation will be reduced to a value which is $\frac{1}{\sqrt{10}}$ times that of an observation from a sample of two apples.

The values for columns 14 to 21 of Table III for a sample of N apples may be found by multiplying the values given in the table by $\frac{1}{\sqrt{N/2}}$. It will be seen that the S.D. of an observation from a sample of 20 apples is relatively small. If the standard size of the sample were increased to such a great extent, it would, of course, be desirable to redetermine by experiment the value of the S.D.

Summary.

1. Commencing with the estimation of acetaldehyde and ethyl alcohol in purely physical systems, and then passing to biological systems, a technique has been evolved which is applicable to the assay of these substances in apples.

2. The standard deviations of the method described have been determined.

3. The method finally adopted is fairly rapid, and its accuracy is sufficient for the work reported in previous papers.

4. In brief, the method consists of a preliminary steam-distillation for 2 hours to rid the fruit of ethyl alcohol and acetaldehyde. Half the distillate is then oxidised to acetic acid, giving a measure of the alcohol number, while the acetaldehyde in the other half is estimated by Ripper's method. The ethyl alcohol figure is found by subtraction.

5. Apples should be distilled as soon as possible after removal from experimental conditions, as otherwise losses of zymasic products may occur (see Appendix I).

same population of apples differing by more than the percentage values given in this column. The well-known expression for this is $2\sqrt{2}$ (S.D.).
(c) All experiments were carried out at 25°.

In addition to the acknowledgments to Mr Meirion Thomas, the Department of Scientific and Industrial Research, and to Armstrong College, which have been made previously [Fidler, 1933, 1], I wish to record my indebtedness to Mr J. Wishart, Reader in Statistics in the University of Cambridge, for his helpful criticisms of the methods of calculation of the standard errors.

Appendices.

THE RELATION BETWEEN THE PRODUCTION OF ACETALDEHYDE AND ETHYL ALCOHOL DURING THE COURSE OF THE EXPERIMENT AND THE AMOUNTS FOUND IN THE FRUIT AT THE TIME OF ANALYSIS.

The alcohol number of apples at the end of an experiment, determined by analysis, is not a true measure of the production of ethyl alcohol and acetaldehyde during the course of the experiment, especially when the analysis is deferred until some time after the fruit has been removed from the experimental conditions.

It has been shown that acetaldehyde disappears from apple tissue on exposure to air[1]. The results of experiments demonstrating this are given in Appendix I.

Ethyl alcohol is transpired by apples, but the loss from the fruit may be measured. The method of estimation and the magnitude of the escape are given in Appendix II.

APPENDIX I.

The loss of acetaldehyde from apples exposed to air.

The work described in this appendix was carried out solely with a view to determining how long analyses of apples could be delayed at the end of an experiment without affecting the acetaldehyde content.

The results may later be useful in considering the metabolism of acetaldehyde[2].

1. *Method.*

In many cases, a definite amount of acetaldehyde solution (standardised by Ripper's method) was added to a known weight of sliced apple tissue, the acetaldehyde content of which had been determined. In the majority of the experiments, the acetaldehyde was formed in the apples by exposing them to nitrogen or carbon dioxide-oxygen mixtures as described by Thomas [1925]. Analyses were carried out on samples immediately on re-exposure to air, and after different periods of aeration. All analyses of fruit were carried out by steam-distillation followed by acetaldehyde estimation by Ripper's method.

2. *Results.*

The results of the investigation are given in Table IV.

It is to be concluded from Table IV that loss of acetaldehyde may occur from apples exposed to air, and that this loss may be serious if the aeration is prolonged. Therefore analyses of apples for acetaldehyde should be carried out as soon as possible after removal from experimental conditions.

[1] Experiments by Thomas [1925] indicated that there was no loss of ethyl alcohol after several days' exposure to air, but work now being carried out seems to suggest that disappearance of ethyl alcohol may take place in special circumstances.

[2] The oxidation of acetaldehyde is one of the aspects of the current researches in this series. It is for this reason that a discussion of the cause of the acetaldehyde loss is not entered upon here.

Table IV. *The change in concentration of acetaldehyde in apple tissue.*

Variety of apple	State of apple	Condition of exp.	No. of hours during which mixture of acetaldehyde and apple tissue was exposed to air	Amount of acetaldehyde present in system (g.)		Percentage loss of acetaldehyde	Whether or not the loss is a significant one
				At beginning of exp.	At end of exp.		
York Imperial	—	Acetaldehyde added in soln.	0·5	0·0092	0·0093	Nil	—
,,	—	,,	0·5	0·0305	0·0277	9·1	Yes
,,	—	,,	0·5	0·0183	0·0175	4·5	No
,,	—	,,	0·5	0·0124	0·0117	5·6	No
,,	Senescent	,,	24	0·0266	0·0205	23·0	Yes
,,	,,	,,	44	0·0325	0·0166	49·0	Yes
Newton Wonder	Young	,,	48	0·0362	0·0334	8·5	Yes
,,	,,	Acetaldehyde formed in N_2	72	0·0033	Nil	100·0	Yes
,,	4 months in store	Acetaldehyde formed in 70 % CO_2·30 % O_2	97	0·0034	0·0001	97·0	Yes
,,	,,	,,	97	0·0046	0·0012	74·0	Yes
,,	5 months in store	,,	72	0·0104	0·0085	18·2	No
,,	,,	,,	72	0·0225	0·0136	39·1	No
,,	7 months in store	,,	48	0·0275	0·0111	59·6	Yes
Winesap	—	,,	72	0·0140	0·0106	24·3	No
Lord Suffield	Very young	,,	73	0·0320	0·0147	54·0	Yes
,,	,,	,,	101	0·0097	0·0034	64·9	Yes

Appendix II.

Transpiration losses.

A. *Ethyl alcohol.* Earlier workers on the subject of carbon dioxide : alcohol ratios realised that the experimental material may lose ethyl alcohol by transpiration. Kostytschew in his text-book [1927] cites the use of an ice-cooled wash-bottle containing distilled water to trap ethyl alcohol. In the researches already described in this series, concentrated sulphuric acid has been used successfully for this purpose.

1. *Method.*

As described in an earlier paper [Fidler, 1933, 1], the gas stream issuing from the fruit chamber passed through a wash-bottle containing 50 ml. of concentrated sulphuric acid. This treatment was shown to rid the gas of all appreciable amounts of ethyl alcohol, the method being checked as follows. A solution of ethyl alcohol in water was standardised by the oxidation method, and definite volumes were measured out into wash-bottles. Air was bubbled through each of these bottles at about 3 litres per hour, for definite time periods at room temperature (12–15°). The issuing air passed through each of two wash-bottles containing 50 ml. of concentrated sulphuric acid. The results are given in Table V.

Table V shows that all the ethyl alcohol escaping from the solution is trapped in the first wash-bottle containing sulphuric acid. The original Dox and Lamb [1916] aeration method of estimating ethyl alcohol made use of the formation of the non-volatile ethyl hydrogen sulphate in this manner.

Table V. *Trapping of ethyl alcohol in sulphuric acid.*

Amount of ethyl alcohol in wash-bottle (g.)		Loss of ethyl alcohol from wash-bottle (g.)	Period (days)	Ethyl alcohol trapped in sulphuric acid (g.)		Net loss or gain of ethyl alcohol (g.)
Beginning of aeration	End of aeration			First wash-bottle	Second wash-bottle	
0·1880	0·1022	0·0858	3	0·0853	Nil	−0·0005
0·1880	0·1290	0·0590	3	0·0605	,,	+0·0015
0·1880	0·1650	0·0230	1	0·0230	,,	Nil
0·1880	0·1650	0·0230	1	0·0248	,,	+0·0018
0·1880	0·0924	0·0956	5	0·0943	,,	−0·0013
0·1880	0·0819	0·1061	5	0·1055	,,	−0·0006

2. *The ratio alcohol content : alcohol escape.*

There is no need to give a detailed account of the magnitude of the escape of ethyl alcohol from apples, but a few examples will be of interest in indicating the values we have observed.

In our experiments on Newton Wonder and Bramley's Seedling apples at 23° during 1930–33, the mean value for the transpired alcohol was 8 % of that contained in the apple. During November and December 1933, an experiment was carried out on Sturmer Pippins in nitrogen at 20°. When the ethyl alcohol content of the tissues had risen to 0·2 % (after 12 days) the escape totalled 0·006 %, but when the alcohol content reached a value of 0·6 % (after 45 days) the escape totalled 0·076 %, or 15 % of the content. This figure has been exceeded with Bramley's Seedling apples.

Thus large errors may be introduced if no account is taken of ethyl alcohol loss by transpiration.

B. *Acetaldehyde.* We have, as yet, few figures for acetaldehyde escape, but such as we have indicate that there is little probability that appreciable error would be introduced into the alcohol figures by this means.

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CL. THE EFFECT OF THE OXIDATION-REDUCTION POTENTIAL OF THE MEDIUM ON THE GROWTH OF TISSUE CULTURES.

By ROBERT EMLYN HAVARD
AND LAURENCE POLLARD KENDAL.

From the Department of Experimental Pathology and Cancer Research, The University of Leeds.

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THE peculiarly high aerobic and anaerobic glycolysis of malignant cells suggests that they may be able to survive conditions of anoxaemia which are fatal to normal tissue cells. Warburg *et al.* [1924] have suggested the possibility of a connection between the increased power of glycolysis shown by tumour cells and their irregular and unrestrained growth. It becomes therefore of some importance to know how normal and malignant cells behave when given a poor oxygen supply while growing *in vitro*. The point has been studied by several workers, but their results have been conflicting.

Wind [1926] found that the Rous chicken sarcoma could grow under strictly anaerobic conditions which inhibited the growth of 10-day chick embryo heart tissue. Wright [1928] also found that malignant cells grew and divided at lower oxygen pressures than that in which normal embryonic tissue could grow; but he did not find that malignant cells would grow under strictly anaerobic conditions. He gives the lowest partial pressure of oxygen at which mitosis occurred in each tissue he examined, as follows:

	mm. O_2
Embryo chick heart	12
Sarcoma (J.R.S.)	6
Carcinoma (M 2164)	3

Fischer [1928] came to the opposite conclusion and reports that malignant cells survived less well under low pressures of oxygen when compared with normal cells.

While our experiments were in progress Laser [1933] published a paper in which he stated that if a culture of normal embryonic tissue were first allowed to grow for 24 hours in a normal atmosphere, it would then continue to grow well for several days under strictly anaerobic conditions. One or two experiments made to test this, reported later in this paper, do not support his conclusions.

It occurred to us that possibly the growth and division of tissue cells in such experiments might be governed more directly by the oxidation-reduction potential of the culture medium than by the concentration of oxygen in the gas phase. That the oxidation-reduction potential of a culture medium might have a real biological significance is suggested by the studies of Knight and Fildes [1930]. They found that germination of the spores of tetanus bacilli occurs only on the negative side of a certain very definite oxidation-reduction potential of the culture medium ($E_h = 110$ mv., when the p_H of the medium $= 7{\cdot}6$).

The E_h of the culture medium they employed fell spontaneously when a stream of purified nitrogen was bubbled through it. They were able to maintain the E_h at any required level by mixing with the purified nitrogen varying proportions of unpurified nitrogen, containing traces of oxygen. The partial pressure of oxygen which would be theoretically in reversible equilibrium with such a solution, $E_h = +110$ mv., would be about 10^{-44} atmospheres [Clark, 1928; Michaelis, 1930]. It is obvious therefore that the oxygen used in these experiments was not in reversible equilibrium with the oxidation-reduction systems of the medium. The definite and consistent results obtained by Knight and Fildes suggest that the germination of tetanus spores took place when a certain level of E_h was reached by the oxidation-reduction systems in the medium, rather than when a particular low concentration of oxygen was present. It is possible that at low oxygen pressures tissue cells also are affected more by the E_h of the culture medium in which they grow than by the oxygen pressure of the gas or of the solution. We have indeed no direct evidence that this is so; the possibility stimulated the performance of this series of experiments.

The purpose of this paper is to record how the growth and division of cells from the embryo chick heart may be correlated with the E_h of the medium in which it is suspended. In a later paper we hope to present a similar correlation between the growth of tumour cells and the E_h of the culture medium.

Experimental.

Preparation of medium and cultures.

In the earlier experiments the salt solution used as the basis of the medium was the phosphate solution recommended by Pannett and Compton [1924]. This fluid was chosen because if Tyrode solution containing bicarbonate were employed, then on passing a stream of nitrogen or air through the medium a considerable quantity of CO_2 would be removed, and the p_H would move too much towards the alkaline side. Media containing the correct concentration of Ca^{++} cannot however be adequately buffered by phosphate, on account of the precipitation of calcium phosphate. The addition of serum also complicates matters. In the later experiments, therefore, the usual Tyrode solution was used, and special gas mixtures containing 2·5 % carbon dioxide were employed. This amount of carbon dioxide kept the p_H of the medium at about 7·4, and in this respect much better control of the conditions was obtained. This change in the nature of the medium did not produce any apparent change in the behaviour of the cultures during an experiment.

Serum was obtained by allowing blood taken from the wing vein of a fowl to clot and the clot to contract and express the serum at 38°. The serum was centrifuged before use. Embryo extract was prepared from a fine mince of two 11-day or three 10-day chick embryos by the addition of 10 ml. Tyrode solution followed by centrifuging. The tissue used was taken from the apex of the heart of a 10–11-day chick embryo. Small fragments were explanted on to $\frac{3}{4}$ in. coverslips in a thin film of a mixture of equal parts of fowl plasma and embryo extract, and this mixture was allowed to clot. 15 ml. of a solution made up of 25 % embryo extract, 10 % serum and 65 % Tyrode solution were placed in a small beaker 6·5 cm. high and with a diameter of 3·5 cm. Three of the coverslip cultures were then placed in the beaker, standing on their edges on the bottom and leaning against the sides in an almost vertical position. The beaker was then closed by the rubber bung carrying the electrodes, gas

inlet and outlet tubes and agar bridge. The whole of the above process was carried out with sterile fluids and apparatus and under strictly aseptic conditions. The bung with its appurtenances was autoclaved while supported in the mouth of a boiling-tube half-filled with distilled water. Just before placing the bung in the culture vessel the tip of the tapered end of the agar bridge, which had been sealed, was broken off so that the KCl-agar might make contact with the medium in the beaker. The outer ends of the gas inlet and outlet tubes were tightly plugged with cotton wool (Fig. 1).

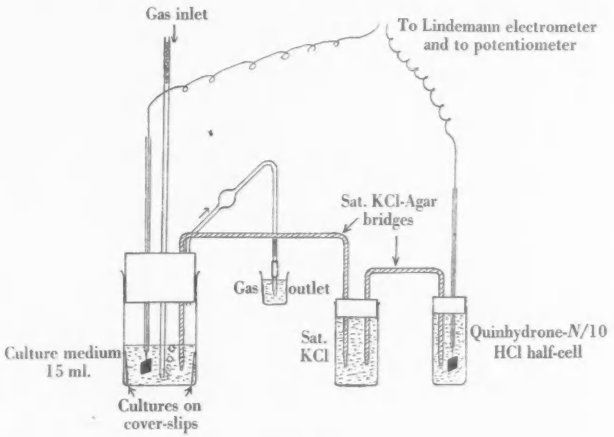

Fig. 1.

Preparation of the gas stream.

Nitrogen containing 2·5 % carbon dioxide was obtained from the British Oxygen Co. This mixture contained about 0·2 % of oxygen, which was removed by passing the gas through two sintered glass wash-bottles containing a solution of chromous chloride. This has the advantage of absorbing the last traces of oxygen without affecting the carbon dioxide [Warburg and Christian, 1931]. Unpurified nitrogen and 2·4 % carbon dioxide from another cylinder were added to the stream of purified gas as required for purposes of poising the potential. A mixture of air with 2·5 % carbon dioxide was passed through the control vessel. With this amount of carbon dioxide the p_H of the media remained at about 7·4.

Both gas mixtures were passed through wash-bottles containing 0·9 % NaCl, which were kept at body temperature in the incubator. The gas was thus saturated with water vapour, and all danger of evaporation of the media was avoided. An open side-tube, at a point just before the gas entered the experimental vessel, dipped into water and served as an escape valve. The pressure of gas passing through the culture vessel could be nicely adjusted by varying the depth of the water. Rubber connections were reduced to a minimum and made where necessary with short pieces of pressure tubing.

Electrical measurements.

The E_h of the culture medium was measured by determining the potential set up on metal electrodes immersed in the solution. The circuit was completed by a half-cell formed by a bright platinum electrode immersed in $N/10$ hydrochloric acid saturated with quinhydrone; the half-cell was connected to the culture vessel by a saturated KCl-agar bridge. The quinhydrone $N/10$ HCl half-cell was used because it is easy to prepare and it will give a potential which is reproducible with considerable accuracy [Morgan *et al.*, 1931]. The potential of this half-cell was taken to be +623 mv. [Clark, 1928, p. 420]. The use of a saturated KCl-agar bridge introduced unavoidable errors of 2–3 mv. [Maclagan, 1929], but this is not serious for experiments involving differences of the order of 10 mv. The metal electrodes were carefully cleaned with hot chromic acid before each experiment by the method described by Morgan *et al.* [1931]. This was very necessary to ensure consistent behaviour of the electrodes in such poorly poised solutions as the media used. It is important to avoid taking any current from the metal electrodes, which are very easily polarised especially in poorly poised solutions. For this reason the use of a static electrometer is essential. The Lindemann electrometer proved to be very convenient and satisfactory. It was set up in the form used by Kerridge [1926] for measurements with the glass electrode. Two or more independent metal electrodes were used in each vessel. An individual electrode, apparently perfect, will sometimes be very inaccurate in these poorly poised solutions. It then has to be discarded.

In the early experiments both gold and platinum electrodes were used in each vessel. Dixon and Quastel [1923] have pointed out that in some sulphur-containing oxidation-reduction systems gold and platinum electrodes may behave differently. In our experiments the gold and the platinum electrodes behaved alike showing that the readings were not complicated by the presence of the anomalous systems described by Dixon and Quastel. In the later experiments only platinum electrodes were used. The electrodes were formed of thin platinum foil, 2 cm.^{2} in area, which were welded on to a short piece of platinum wire. The platinum wire was fused on to a piece of copper wire and was then fused into a glass holder with sealing-in glass.

In the early experiments a glass electrode (Haber's bulb form) was also immersed in the culture medium. The bulb of the electrode was blown from the special glass recommended by MacInnes and Dole [1930]. It was obtained from the Corning Glass Co., New York (No. 015). The glass electrode contained $N/10$ HCl and was fitted with a $N/10$ HCl quinhydrone half-cell. Special precautions were taken to ensure perfect insulation. By this means it was possible to determine whether there was any gross change of reaction of the culture medium during the experiment. If bacterial infection developed the medium turned rapidly acid (at the same time the E_h would be rapidly lowered), but in an uninfected culture the p_H remained practically constant for the duration of the experiment. Therefore, for the sake of simplicity, the glass electrode was discarded in the later experiments. The p_H of the medium was determined at the end of each experiment. In an uninfected experiment, in which the 2·5 % carbon dioxide mixtures were used, the p_H was always about 7·4. In an infected culture it had often fallen to below 7·0.

On passing the oxygen-free nitrogen and carbon dioxide mixture through the culture medium, the potential of the metal electrode, and therefore the E_h of the solution, fell more or less rapidly. If the unpurified nitrogen mixture was added to the gas stream as the required potential was approached the fall in E_h

was checked. By varying the rates of flow of the oxygen-free and the unpurified mixtures the E_h could be poised at the required level. The culture medium in this respect behaved like the media used by Knight and Fildes [1930].

Experimental procedure.

The cultures and electrode vessels, having been assembled, were placed in the incubator, and the electrical connections were made to the electrometer leads and to the standard quinhydrone half-cell. After allowing 30 mins. to 1 hour for temperature equilibration, the first potential measurements were made. The gas flow was then started; through the experimental vessel O_2-free nitrogen with 2·5 % CO_2; through the control vessel air with 2·5 % CO_2.

It was found that violent bubbling of gas through the fluid medium had for some unknown reason a deleterious effect upon the cultures. The reason for this is not clear, but it may be related to the fact that the growth-promoting power of embryo extract [Carrel, 1913] and of serum [Carrel and Ebeling, 1922] is said to be diminished by shaking. Whether this is an oxidation or merely a mechanical inactivation is not known. It was necessary, however, because of this phenomenon, to allow only a short spell of rapid bubbling to remove oxygen from the fluid and space above it, and then to cut down the rate of flow until the amount of agitation of the fluid was quite small. Throughout the experiment care was taken that the rates of flow of gas through experimental and control vessels were, as far as could be judged, the same. When the potentials registered by the electrodes in the experimental vessel had fallen almost to the desired level, unpurified N_2, *i.e.* N_2 containing a small quantity of oxygen, was introduced into the gas stream, and the rates of flow of purified and unpurified gas were adjusted so that the potentials came to and remained at this level. The experiment was continued for 40–48 hours, the gas flow in the experimental vessel being adjusted when necessary to maintain the constant potential. After 40–48 hours the coverslips were taken out of the vessels and the cultures, experimental and control, fixed in Bouin's fluid, stained with Mayer's haemalum, and examined The p_H of the fluid medium was determined, at 38°, with the aid of a glass electrode of the Stadie type [Stadie *et al.*, 1931].

Results.

Fig. 2 shows the potentials which were observed in a typical experiment. Each curve relates to a single electrode, and in this experiment the gas flow was adjusted to maintain the E_h indicated by the electrodes in the experimental vessel at about 20 mv. It will be noted that the variation between the potentials of the three electrodes in the experimental vessel covers a range of about 20–30 mv., which is sufficiently small for the purposes of this work. In the control vessels the agreement is not quite so good. This might be expected where a considerable amount of oxygen is present. During the first 24 hours of the experiment there is a gradual drift of the potential to a more positive value. These characteristics were observed so consistently in all the experiments that in many of the later ones it was not considered necessary to make potential measurements in the control vessel.

Experiments were discarded in any of the following circumstances. (1) When there was marked disagreement between the behaviour of the electrodes in the same vessel. (2) When the E_h during the course of the experiment showed wide fluctuations owing to imperfect adjustment of the gas stream. (3) When the culture medium showed evidence of bacterial infection. (4) If for any unknown reason the control cultures did not grow well.

The following experiments we consider to be free from these defects: two in which the E_h was maintained at +120 mv., one in which it was maintained at +20 mv., one at −30 mv., and two at −100 mv. An indication of the activity of the cultures may be obtained by reference to Table I. To obtain the

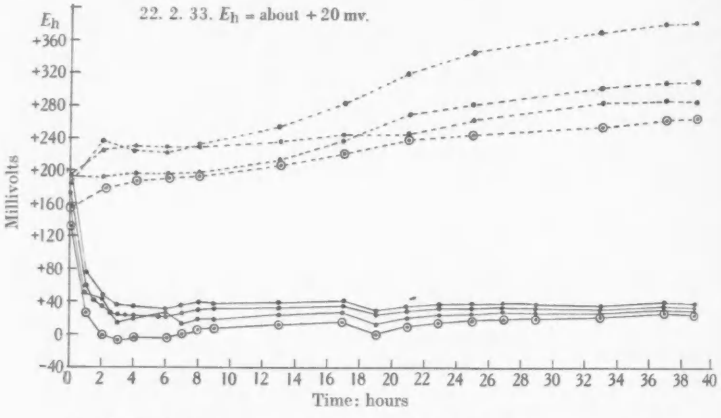

Fig. 2. Continuous line, experimental vessel. Broken line, control vessel. •—•, Platinum electrodes. ⊙—⊙, Gold electrodes.

Table I.

No. of exp.	E_h of medium (mv.)		Areas in mm.² Area of explant A_1	Area of whole culture A_2	Area of outgrowth $A_2 - A_1$	Mean area of outgrowth of exp. culture as % of that of controls
1	+120	Exp.	0·27	1·87	1·60	
			0·34	2·38	2·04	34
		Contr.	0·42	5·57	5·15	
			0·37	5·85	5·48	
2	+120	Exp.	0·58	2·63	2·05	
			0·67	2·73	2·06	
			0·57	2·23	1·66	43
		Contr.	0·33	4·78	4·45	
			0·57	5·04	4·47	
3	+ 20	Exp.	0·44	1·07	0·63	
			0·45	1·91	1·33	12
		Contr.	0·54	7·62	7·08	
4	− 30	Exp.	0·64	1·06	0·42	
			0·69	0·81	0·12	2·3
		Contr.	2·34	15·25	12·91	
			1·95	13·05	11·10	
5	−100	Exp.	Only a few cells have migrated out			
		Contr.	0·93	5·93	5·00	
			0·97	4·72	3·75	
			0·86	4·96	4·10	
			0·58	3·38	2·80	
6	−100	Exp.	Only a few cells have migrated out			
		Contr.	Cultures infected			

figures recorded in this table the fixed and stained cultures were placed under the microscope, and with the aid of a drawing eye-piece the outlines of the central fragment and of the whole culture were traced on to a sheet of paper. The areas outlined on the paper were then measured with a planimeter, and knowing the magnification, the actual areas of the cultures were calculated. These are given in the table in mm.^{2} For the purpose of comparing the growth of experimental and control cultures the area of the outgrowth has been used. This is given by the difference between the area of the whole culture, A_2, and that of the central fragment, A_1. It was thought that this would be a truer representation of the activity than the fraction A_2/A_1 which is often used. A_1 in the latter expression ought really to be the original area of the explant, but the area of the explant when measured at the end of the experiment is sometimes, especially in well-growing cultures, considerably greater than its initial value. This is very noticeable in Exp. 4 in the table. When the difference A_2-A_1 is considered, however, the error due to this uncertainty about the true original area is much diminished, because A_1 is small compared with A_2. The final column of the table shows the average outgrowth of the experimental cultures expressed as a percentage of that of the control. It should be pointed out that in addition to diminution in the area of outgrowth in the series, there is at the same time a very marked decrease in the density of cell population in the outgrowth. This factor cannot conveniently be gauged numerically, but because of it the nett effect of reducing the E_h of the medium is really much more marked than the figures in the table suggest.

The apparent E_h in the control vessels was always of the order of +280 to +360 mv. When the E_h was maintained at +120 mv., the activity of the cultures was already markedly inhibited but was still considerable. In the two experiments the areas of outgrowth were 43 and 34 % respectively of those of the control cultures. Mitoses were still occurring at this E_h, as many as 19 being observed in a single culture. Examples can be seen in Plate VI, fig. 1. The appearance of the cells was not different from that of the control seen in Plate VI, fig. 2, though the number of mitoses was much smaller.

At an E_h of +20 mv. a much greater inhibition of activity was evident. The area of outgrowth was only 12 % of that of the controls and was more sparsely populated with cells. The condition of the cells was still fairly good, and there were one and four mitoses respectively in the two cultures (Plate VI, fig. 3). One or two of these appeared somewhat abnormal.

At −30 mv. the outgrowth was even smaller, only 2·3 % of that of the controls, but it cannot be said that activity was completely inhibited. There were no mitoses; the cells were heavily vacuolated and appeared to be on the verge of degeneration (Plate VI, fig. 4).

At −100 mv. only a few cells had migrated from the explant and it seems possible that this migration had occurred in the early stages, before the strongly reducing character of the environment had had time to overcome some innate capacity of the cells for resisting. Nevertheless the condition of the cells in the experimental cultures (Plate VI, fig. 5) appeared to be better than that shown in Plate VI, fig. 4. The reason for this is at present obscure.

The general result was therefore that when the potential indicated by the electrode was +20 mv. or more positive a considerable amount of activity was permitted. Even at a potential of +120 mv. there was a marked inhibition of growth, and this inhibition had reached a maximum at −30 mv. There was little difference, if any, between the amount of activity at −30 mv. and that at −100 mv. Even at these low levels some of the cells retained a limited power of

migration for a few hours at any rate, and did not appear to be killed within the time of the experiment.

Inhibition of mitosis appears to be complete at a potential which is rather more positive than that necessary for maximum inhibition of migration. Mitosis has almost completely ceased at +20 mv., and was not observed at −30 mv.

In some of the experiments at potential levels below +120 mv., the cells of the experimental cultures showed heavy vacuolation and distortion of nuclei suggesting imminent degeneration. These appearances were not however sufficiently regular to be related to the potential level. They must at present be ascribed to some unknown and as yet uncontrolled variable in the environment of the cultures.

It has been mentioned that Laser [1933] has recently stated that chick embryo tissue can grow equally well under aerobic or anaerobic conditions, but only when it has first been allowed to grow under normal conditions for 24 hours. Two experiments have been made to test this. The experiments were carried out in the same way as those described above, but the vessels with the cultures were left in the incubator undisturbed for 1–2 days before the flow of nitrogen was commenced. The experimental cultures were then allowed to grow for a further similar period of time at $E_h + 50$ mv. The two experiments produced identical results. Fixing and staining of the cultures showed that the growth of the experimental cultures had been markedly inhibited, and the cells were much more heavily vacuolated than were the controls. There were several mitoses, but far fewer than in the controls. In many the mitotic figure was distorted in a striking fashion by the large vacuoles (probably fat) in the cytoplasm (Plate VI, fig. 6). In spite of the preliminary growth under aerobic conditions therefore the effects observed fit very well into the series of experiments already described and correspond with what was expected at that level of potential. Thus these results do not support Laser's conclusions.

Discussion.

In the experiments described above mitosis was observed when the oxygen pressure must have been considerably lower than Wright's minimum figure for the same type of tissue, *i.e.* 12 mm. Hg. This follows from the fact that the oxygen pressure in the unpurified nitrogen was only about 2 mm. Hg, and this was diluted by oxygen-free nitrogen before reaching the cultures. Mitosis therefore occurred when the oxygen pressure was much below 2 mm. Hg.

In considering these experiments, it is important to realise that the oxidation-reduction systems which determine the electrode potentials cannot be in reversible equilibrium with the oxygen of the gas phase. The arguments which apply to the experiments of Knight and Fildes were supported by our experience. An E_h of +100 mv. corresponds thermodynamically to an oxygen pressure of about 10^{-45} atmospheres. To maintain this E_h it was necessary to pass in a certain proportion of unpurified nitrogen containing about 0·2 vol. % of oxygen. Although the final composition of the gas phase was not determined it must, of course, have been far in excess of 10^{-45} atmospheres.

These considerations, together with the fact that the electrode potential can be raised or lowered by corresponding changes in the oxygen content of the solution, suggest that we may be dealing with a complex system somewhat as follows. We suggest, firstly, that the medium contains several reversible oxidation-reduction systems of an unknown nature. Each system is able to poise the potential, in the range of E_h about its own E_0 value, to an extent dependent upon the amount of the particular system present. The range of E_h covered by

all the systems together is such that some degree of poising can be manifested at any potential between, at any rate, +200 mv. and −100 mv. Secondly, the medium contains a reducing substance, or substances, which in the absence of oxygen can reduce the oxidised component of any of the reversible systems which determine the electrode potentials. In the absence of oxygen therefore this reduction takes place and the potential falls to a very low figure. Thirdly, in the experiments described, the medium contains oxygen. This, while not itself establishing equilibrium with the electrodes, tends to oxidise those systems which are in such equilibrium and therefore to raise the potential. A combination of these three factors might be expected to produce the observed results. The potential is set up by a reversible oxidation-reduction system effective at that point in the E_h scale. The condition of the reversible system is determined by the balance between oxidation of the system by oxygen and reduction of the system by a more strongly reducing substance in the medium.

The vigour of the reducing substances appears to vary in different samples of serum and embryo extract. In one experiment the E_h fell rapidly in the absence of oxygen to below −400 mv. In another experiment in which the same precautions were taken to eliminate traces of oxygen, the E_h did not fall below −20 mv. These results suggest that perhaps other factors besides oxygen pressure should be taken into account when considering the growth of tissues in low concentrations of oxygen. It is possible that the growth may be affected by the E_h more than by the oxygen pressure. The question, which of these two is the more important, cannot yet be regarded as settled. The possibility still remains that, whatever may be the factors governing the E_h, the growth of tissue is after all determined directly by the concentration of oxygen. More work upon the growth of cells under limiting conditions of oxygen pressure and of E_h is needed before this point can be decided.

It is of interest to consider these results in relation with those obtained by Needham and Needham [1925] and by Cohen *et al.* [1928], who studied the E_h of the protoplasm within amoebae by the micro-injection of oxidation-reduction dyes. From the results given by Needham and Needham the E_h within an amoeba appears to be maintained at about +100 mv. The experiments recorded above show that embryonic tissue cells, at any rate, can not only migrate but show mitosis when the medium surrounding them has E_h as low as +20 mv. It would be surprising if cells were able to live and multiply in a medium with E_h 80 mv. more negative than that within the cell itself. Our results are more in accord with those of Cohen *et al.*, which suggest that the E_h of the interior of the cell is below −130 mv. With E_h −100 mv. we find that embryonic cells of the chick are still able to survive to some extent, though there is no mitosis and little or no migration.

Summary.

1. On passing pure nitrogen through a culture medium containing serum and embryo extract the E_h of the medium fell rapidly.

2. By adding different small amounts of oxygen the E_h could be maintained at any desired level between +300 mv. and −100 mv.

3. The growth and division of cells from embryonic chick heart tissue have been studied when the E_h of the medium has been maintained at different levels. In general the lower the E_h the poorer has been the growth of the tissue culture.

4. All mitosis ceased between E_h + 20 mv. and −30 mv. Migration of cells had almost entirely ceased at an E_h of −30 mv.

5. The possible significance of these results is discussed.

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DESCRIPTION OF PLATE VI.

Fig. 1. Part of culture in medium at $E_h = 120$ mv., showing mitosis. ×400.

Fig. 2. Part of control to Fig. 1. ×400.

Fig. 3. Culture grown with medium at $E_h = 20$ mv., showing mitosis. ×400.

Fig. 4. Culture grown with medium at $E_h = -30$ mv. ×400.

Fig. 5. Culture grown with medium at $E_h = -100$ mv. ×400.

Fig. 6. Culture grown in air for 36 hours, then at $E_h = 50$ mv. for 48 hours. ×400.

Note distorted mitotic figure in the centre of the field. The equatorial plate of chromosomes is pushed towards one end of the cell and is no longer lying at right angles to the long axis of the cell, as the result of the presence of large vacuoles, probably representing fat.

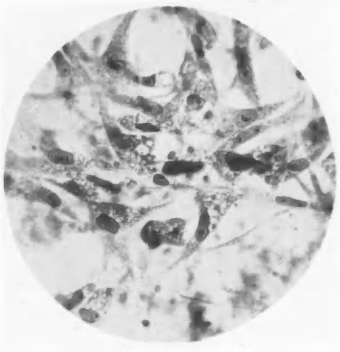

Fig. 1.

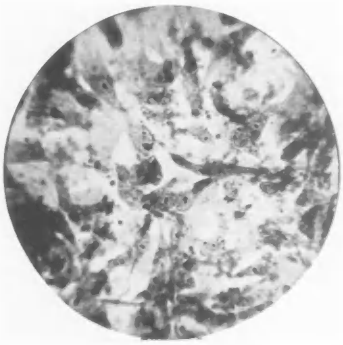

Fig. 2.

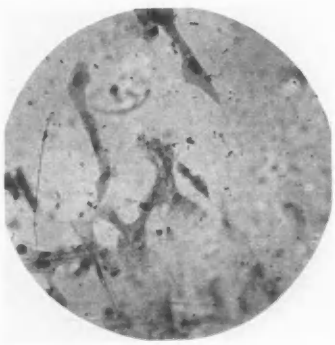

Fig. 3.

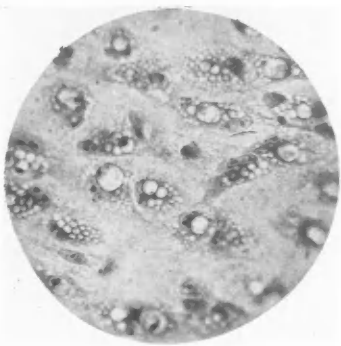

Fig. 4.

Fig. 5.

Fig. 6.

UNIV. OF MICH.

CLI. THE PRESENCE OF A SAPOTOXIN IN *XANTHOSOMA ATROVIRENS*, A TROPICAL FOOD-TUBER.

By ALFRED CLARK and ROY BASIL WATERS.

(*Received March 24th, 1934.*)

It has long been known that certain plants containing glucosides of the saponin group cause much disease and high mortality amongst domestic animals in different parts of the world [Ewart, 1931]. It has not however been shown previously that this class of poisons is present in harmful quantities in any plant used in human consumption, excepting perhaps the glyco-alkaloid in the potato (*Solanum tuberosum*) and the sapotoxin of *Agrostemma githago* seeds which are sometimes accidentally present in wheat and ground with it in milling.

The tubers of several plants of the natural order Araceae are largely consumed in tropical countries, and an account of the extraction from the one most extensively eaten in the West Indies of an acid sapotoxin which produces nephritis is of interest, especially in view of the heavy incidence of this disease amongst the poorest class of the population in Trinidad and other tropical countries.

The tuber selected for special examination is the Tannia (*Xanthosoma atrovirens*) as this is eaten all the year round whilst most of the others have their season. The wide adoption of tannia as a food is explained by the fact that it is palatable, abundant and cheap. Further, apart from its unfortunate toxicity, it has a fairly satisfactory composition as a foodstuff. During an investigation of its dietetic value it was found that the addition of cholesterol or of sterol-containing foods to a tannia diet materially lessened its toxic effects. This suggested the presence of a sapotoxin and attempts were made to isolate such a substance.

The peeled tubers were finely minced and mixed with distilled water. After several hours an equal volume of alcohol was added and the precipitated starch removed by filtration. The filtrate was freed from most of the alcohol by evaporation on the water-bath, and the hot solution was treated with 30 % normal lead acetate. A precipitate was formed and collected, but the filtrate retained some of its toxicity. Treatment with basic lead acetate yielded a further smaller precipitate and the filtrate from this, after removal of lead, was found to be free from toxic substance.

The normal lead acetate precipitate, after washing with distilled water, was suspended in water and treated with hydrogen sulphide. The resulting lead sulphide appeared to adsorb much of the toxic material which could not be separated entirely, either by prolonged treatment with hydrogen sulphide or by repeated boiling of the precipitate with water. The filtrate was evaporated under diminished pressure to a syrup from which an excess of cold alcohol precipitated a white hygroscopic solid. This solid had the chemical properties of an acid sapotoxin and showed a high toxicity when its solution in normal

saline was injected intravenously into rats. A large amount of inorganic impurities was present. Dissolution of the solid and repetition of the above processes removed most, but not all, of this impurity.

The alcoholic filtrate, on evaporation, yielded a dark gum. This was dissolved in water and treated with hot baryta solution. The precipitated barium salt was collected and well washed with water, and its suspension in water was treated with 10 % sulphuric acid in slight excess. The excess was neutralised with lead carbonate and the filtered solution evaporated under diminished pressure. The yellow residue was dissolved in alcohol and the solution decolorised with charcoal, filtered and evaporated. The clear gum thus obtained was dissolved in ethyl acetate and the solution filtered from inorganic material and evaporated. Repetition of the treatment with ethyl acetate gave a clear gum which crystallised on standing. Recrystallisation from ethyl acetate gave pure crystals of citric acid, M.P. 152°, either alone or when mixed with an authentic specimen. A solution of this product in normal saline produced little or no discomfort to rats when injected intravenously.

The basic lead acetate precipitate was similarly decomposed by hydrogen sulphide and the filtrate from the lead sulphide evaporated to a syrup. On pouring cold alcohol into this a cream-coloured solid was precipitated. This also proved to be a sapotoxin with an acid reaction and in physiological effect was indistinguishable from that obtained from the normal lead acetate precipitate. It was, however, much smaller in amount than the latter.

The alcoholic mother-liquors of this sapotoxin were evaporated to a gum, which was shown by its chemical and physiological properties to contain a sapotoxin. Various attempts were made to purify this gum, but without complete success. A solution of the material in water was mixed with freshly prepared magnesium hydroxide and evaporated to dryness at 50°, and the resulting mass was powdered and boiled with alcohol to remove any uncombined organic material. The solid was then suspended in water and a rapid stream of carbon dioxide passed through the mixture for several hours. The precipitated magnesium carbonate was removed by filtration and the solution evaporated to small bulk under diminished pressure and poured into a large excess of alcohol. The solid thus obtained was redissolved in a little water and again precipitated with alcohol. This gave a pale brown amorphous powder which still contained magnesium. Its solution in water was treated with basic lead acetate solution, the precipitate collected and well washed. This was decomposed by hydrogen sulphide and the filtrate evaporated to a syrup, which was dissolved in a little alcohol, filtered and diluted with a large volume of ether. This procedure gave a white, amorphous solid which still contained a small quantity of inorganic salts. It was extremely deliquescent, giving a brown gum on exposure to moist air for a few seconds, and possessed the properties of a sapotoxin.

The acid sapotoxins prepared in the above manner were believed to differ only in degree of purity. They were found to contain a little material which reduced Fehling's solution, and this was removed by boiling with alcohol. The product then contained 7 % of ash. Its solution was dextrorotatory and after hydrolysis with dilute sulphuric acid was less strongly dextrorotatory. The hydrolysed solution was neutralised with barium carbonate, evaporated to small volume under diminished pressure and poured into alcohol. A solid, presumably the sapogenin, separated out and was removed by filtration. The alcoholic solution was evaporated to dryness under diminished pressure. The residue reduced Fehling's solution and when heated with strong hydrochloric acid gave a very weak furfuraldehyde reaction to aniline acetate paper. An osazone was

obtained which, microscopically, resembled glucosazone, M.P. 194–196°. Owing to lack of material it was not possible to recrystallise the osazone. When, however, it was mixed with an authentic specimen of glucosazone, the M.P. was raised to 199–200°. The sapotoxin thus appears to contain a hexose which yields glucosazone.

It would be difficult to make even a rough estimate of the toxin content of the tubers owing to the great loss by adsorption during the processes required for its isolation and because the content of the tubers, as indicated by monthly feeding experiments on rats, showed a great variation in the duration of life of animals fed on them. This duration was on an average 4 days in January: 22 days in March, April, May and June: 7 days in the remaining months.

The sapotoxins thus extracted from tannia tubers are highly toxic to rats. Intravenous injection of 0·1 mg. killed a 60 g. rat instantaneously from cardiac and respiratory paralysis: 0·05 mg. killed in 20 minutes. Injection of smaller quantities produced in 50 minutes a rise of blood-sugar ranging from 176 to 415 mg. per 100 ml. At the same time the hepatic glycogen was found to be greatly diminished, usually to the merest trace. The urine, when obtainable, was albuminous. A solution of 1 part of sapotoxin in 20,000 parts of normal saline haemolyses blood *in vitro*, with reduction of the liberated haemoglobin. Spectroscopic examination shows the presence of the bands characteristic of haematoporphyrin. The cholesterol-saponin compound is neither toxic when injected, nor haemolytic.

The raw tubers have a burning effect upon the tongue which is sometimes severe but usually not unpleasant. Rats eat them readily and their average daily consumption when given no other food is 0·35 g. per g. rat, which is slightly more than the amount consumed of the stock diet. In spite of the adequate food consumption the animals lose weight from the commencement of the experiment. Within 24 hours the faeces become putty-like and clay-coloured. The abdomen becomes greatly distended and the urine albuminous. A gradually increasing lethargy follows which becomes profound as death approaches. Adrenaline injected intravenously rouses the animal rather dramatically, but the stupor returns in a short time and a further injection has little or no effect. The blood-sugar in the terminal phase of the experiment is greatly diminished, amounts as low as 45 mg. per 100 ml. being observed, whilst at the same time it is found that the hepatic glycogen is almost or quite absent.

The *post mortem* signs are remarkably constant. The caecum is enormously distended and its walls much thinned. The average weight of this organ in 27 examinations of rats fed on raw tannia was 14·6 % of body weight (one 20·7 %) compared with a weight of 1·47 % in rats fed on normal diet. The stomach and intestines contain blood in almost every case. The liver, lungs and kidneys are usually very dark, the cortex and medulla in the last-named being hardly distinguishable by their colour. Special interest is attached to the condition of the adrenal glands in view of the disturbed carbohydrate metabolism in tannia poisoning. In the normal rat these are of a pale skin colour and, in the albino rat of 60–70 g. body weight, account for about 0·0275 % of body weight, but in rats of the same strain and weight which have been poisoned by tannia are about 0·0390 % and vary in colour from a dull pink to a purplish tint similar to that of the kidney. Microscopically the kidneys show the appearances characteristic of an acute toxic nephritis. The glomeruli and secretory tubules are much disintegrated and the picture presented closely resembles that produced in poisoning by uranium nitrate. Granules of blood pigment are found abundantly scattered in kidneys, suprarenals and spleen and are especially plentiful in the

reticulo-endothelial cells of the liver. These appearances are found whether the tubers have been ingested or the toxin injected, but in the latter case the effects are more marked in the kidneys and less in the other organs than in the former.

Unstained blood films prepared in the ordinary way from rats poisoned by tannia or injected with its toxin show colourless crystals which are quite new in our experience. These are usually regular, stellate structures and many are six-pointed. They are about twice as large as a red blood corpuscle. We have not been able to find them in the films when freshly spread, but they can be discovered an hour later. They are not very numerous, but are striking and characteristic when found. It is not yet known whether they occur in cases of tannia poisoning in human beings in tropical countries. Ordinary haemoglobin crystals are sometimes found in association with the stellate crystals, being produced no doubt by the saponin haemolysis.

SUMMARY.

A highly poisonous acid sapotoxin, for which the name tanniatoxin is suggested, has been isolated from the tubers of *Xanthosoma atrovirens*, a food commonly eaten in Trinidad under the name of "tannia."

When injected in doses of 0·1 mg. it kills a 60 g. rat instantaneously from paralysis of heart and respiration.

In smaller doses or when the raw tubers are eaten, a glomerulo-tubular nephritis is produced.

The sapotoxin haemolyses blood *in vitro* and converts the liberated haemoglobin into haematoporphyrin.

Certain stellate crystals which are found in blood films of poisoned rats are described.

It is suggested that certain cases of nephritis occurring in tropical countries may be caused by the consumption of tannia and other aroid tubers.

We desire to thank the London School of Hygiene and Tropical Medicine for their generous provision of laboratory facilities. We are also indebted to the Medical Research Council for whole time grants which have made the investigations possible.

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CLII. THE EFFECT OF THYREOTROPIC HORMONE AND OF DESICCATED THYROID UPON CREATINE AND CREATININE EXCRETION.

By LEONARD IRVING PUGSLEY, EVELYN M. ANDERSON, and JAMES BERTRAM COLLIP.

From the Department of Biochemistry, McGill University, Montreal.

(*Received April 13th, 1934.*)

Cramer and Krause [1913] observed a creatinuria in both men and dogs as a result of feeding thyroid tissue. Gross and Steenbock [1921] found that feeding sheep thyroid to a pig on a nitrogen-free diet led to an increase in the creatine excretion. Patients with Graves' disease excrete excessive amounts of creatine in the urine. It has been suggested by Shorr *et al.* [1933] that the thyroid hormone is involved in the breakdown and building up of phosphocreatine. Eimer [1931] reported a significant increase in the total creatinine excretion of healthy subjects when fed desiccated thyroid. Recently, Shrire and Zwarenstein [1933] have shown that the administration of anterior pituitary extracts causes an increased excretion of creatinine in normal rabbits but not in castrated animals.

Methods.

Adult rats were placed on the following diet 3 weeks before the metabolism studies were started: wheat flour 1000 g., corn meal 1000 g., oatmeal 1000 g., linseed meal 300 g., wheat germ 82 g., brewer's yeast 182 g., cod-liver oil 180 ml. We have found the diet to be an important factor in studying creatine metabolism. The addition of meat or fish meal to the diet gives relatively high and irregular values for the creatine excretion. The rats were kept in Hopkins's metabolism cages and the urine and faeces collected separately. The urine was collected over a period of 48 hours, and the samples were made up to a volume of 20 ml.; the creatinine was estimated on a convenient aliquot by the method of Folin [1914].

The creatine was calculated by difference after autoclaving the urine in the presence of picric acid, and it is expressed here in terms of creatinine. The oxygen consumption was measured in a multiple chamber respiration apparatus, similar to that described by Benedict [1930–31]. The metabolic rate is expressed as the percentage increase in oxygen consumption over that determined in the pre-experimental period. Since fasting alone has an effect upon the creatine and creatinine excretion, the metabolic rates could not be taken under fasting conditions; however, on a series of several hundred rats we have found that the oxygen consumption, measured under feeding conditions at approximately the same hour each day, tends to be remarkably constant (unpublished data). Hypophysectomy was performed on the rats by the parapharyngeal approach of Selye [Collip *et al.*, 1933]. Two weeks were allowed after the operation before the creatinine studies were started.

The thyreotropic extract which was used showed on assay 100 units per ml. [Anderson and Collip, 1933]. One unit represents the smallest amount of the hormone which, when given twice daily to an hypophysectomised rat, will produce a 20 % increase in oxygen consumption within 96 hours.

Results.

Fig. 1 shows the effect of feeding desiccated thyroid upon the metabolic rate and the creatine and creatinine excretion of normal rats. The values in this chart represent the average of 6 rats. After a control period of 6 days, the rats were each given 2 grains of desiccated thyroid daily in their food. A very striking increase in creatine output occurred, which reached a peak on the twelfth day of thyroid feeding. It then commenced to drop off gradually, in

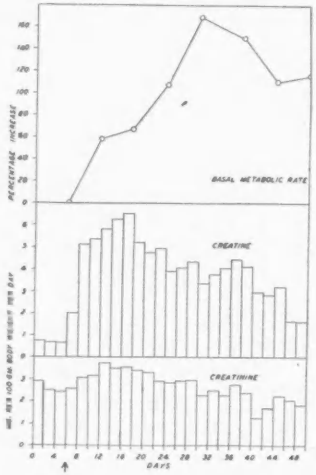

Fig. 1. The effect of feeding 2 grains of desiccated thyroid daily upon the metabolic rate and the creatine and creatinine excretion of adult rats.

spite of a rising metabolic rate. The creatinine excretion was increased slightly during the first 2 weeks of thyroid administration. After 24 days of thyroid feeding the animals were in a poor condition due to the extreme hyperthyroidism, and 3 rats of the series died before the experiment was finished. The drop in the metabolic rate which occurred in the sick rats lowered the average rate for the group.

In another series of experiments, a group of 4 rats was given thyreotropic hormone. The effect upon the metabolic rate and the creatine and creatinine excretion is shown in Fig. 2. Fifty units of thyreotropic hormone were injected intraperitoneally twice daily for a period of 32 days. A rise in the creatine excretion occurred immediately after beginning the injections, and the excretion then gradually fell to the normal level. The metabolic rate rose to a level of + 35 %, and then gradually fell, finally below the pre-experimental level.

Fig. 3 shows the effect of the thyreotropic hormone upon the metabolic rate and the creatine and creatinine excretion of hypophysectomised rats. A group

of 4 animals was studied. The thyreotropic hormone in doses of 50 units twice daily was started on the tenth day. It is seen that the creatine excretion reached a peak by the fourth day of injection and then began to decline while the metabolic rate was still rising. This suggests that the relationship between the two is not as direct as one might conclude from Fig. 2. The amount of creatinine

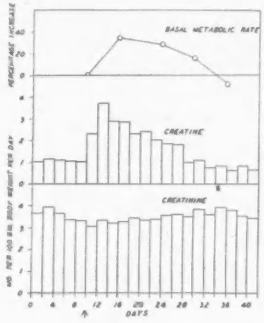

Fig. 2.

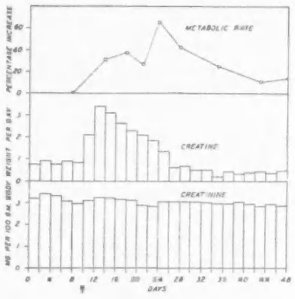

Fig. 3.

Fig. 2. The effect of 50 units of thyreotropic hormone upon the metabolic rate and the creatine and creatinine excretion of adult male rats.

Fig. 3. The effect of 50 units of thyreotropic hormone upon the metabolic rate and the creatine and creatinine excretion of hypophysectomised rats.

excreted remained unchanged throughout the experiment and was essentially the same as that of normal rats. This differs from the findings of Braier [1931], who reported a 35–40 % decrease in the creatinine excretion of hypophysectomised dogs.

The thyreotropic hormone produced a rise in the creatine output of the dog. This is shown in Table I. It will be noted that a diuresis occurred simultaneously with the creatinuria.

Table I. *Creatine and creatinine excretion of a dog treated with thyreotropic hormone. Wt. 26·6 kg.*

	Day	Urine volume ml. per day	Creatinine g. per day	Creatine as creatinine g. per day
Control period	2	867	1·103	0·357
	4	725	0·668	0·195
	6	1135	1·003	0·334
Experimental period. Injections started on 6th day; 500 units thyreotropic hormone twice daily, intravenously	8	1250	0·817	0·807
	10	1335	0·861	0·795
	12	1675	0·885	1·273
	14	1070	0·718	0·968
	16	1375	0·891	0·663
	18	1425	0·789	0·479
	20	1550	0·790	0·603
	22	1375	0·715	0·524
Recovery period. Injections stopped on 22nd day	24	1360	0·743	0·262
	26	1050	0·550	0·088
	28	930	0·756	0·055
	30	1000	0·515	0·110

The effect of small doses of thyreotropic hormone on creatine excretion has been studied. In Table II it is seen that 6 units of thyreotropic hormone are

Table II. *The effect of graded doses of thyreotropic hormone upon the creatine and creatinine excretion of normal rats.*

		Group I (average of 4 rats)			Group II (average of 4 rats)			Group III (average of 4 rats)		
	Day	Creatinine mg.	Creatine mg.	Metabolic rate	Creatinine mg.	Creatine mg.	Metabolic rate	Creatinine mg.	Creatine mg.	Metabolic rate
Control period	2	3·65	1·16	—	2·80	0·58	—	3·08	0·69	—
	4	3·33	1·09	—	3·22	0·64	—	2·96	0·36	—
	6	3·28	1·04	Normal	2·84	0·79	Normal	2·74	0·56	Normal
Injections started on 6th day		Dose: 50 units b.i.d.			Dose: 12 units b.i.d.			Dose: 6 units b.i.d.		
Experimental period	8	3·03	2·17	—	2·94	0·62	—	2·64	0·50	—
	10	3·31	3·71	—	2·81	0·74	—	2·64	0·57	—
	12	3·24	2·78	+34·8 %	2·82	1·20	—	2·73	1·06	—
	14	3·44	2·30	—	3·08	1·20	+17·3 %	2·93	1·27	+28·7 %
	16	3·32	2·41	—	3·03	0·53	—	3·15	0·28	—
	18	3·40	2·06	+28·1 %	3·09	0·44	+16·7 %	3·17	0·34	+19·1 %

The thyreotropic hormone in doses as low as 6 units twice a day causes an increase in creatine excretion in the normal rat. The creatine and creatinine are expressed in mg. per 100 g. body weight per day. The creatine is expressed in terms of creatinine. The "metabolic rate" is the percentage increase in oxygen consumption over that determined in the control period.

sufficient to cause a significant rise in the creatine excretion and metabolic rate of normal rats. In the hypophysectomised rat, 1 unit causes a more striking increase in the creatine output than is produced after 6 units in the normal rat (Table III).

Table III. *The effect of thyreotropic hormone upon creatine and creatinine excretion of hypophysectomised rats.*

		Group I (average of 4 rats)			Group II (average of 2 rats)			Group III (average of 2 rats)		
	Day	Creatinine mg.	Creatine mg.	Metabolic rate	Creatinine mg.	Creatine mg.	Metabolic rate	Creatinine mg.	Creatine mg.	Metabolic rate
Control period	2	3·34	0·77	—	2·85	0·77	—	2·65	0·95	—
	4	3·02	0·90	—	2·79	0·17	—	3·14	0·28	—
	6	2·90	0·83	Normal*	2·81	0·15	Normal*	2·77	0·18	Normal*
Injections started on 6th day		Dose: 50 units b.i.d.			Dose: 2 units b.i.d.			Dose: 1 unit b.i.d.		
Experimental period	8	3·10	2·12	—	2·95	0·14	—	3·00	0·12	—
	10	3·28	3·38	+31·5 %	2·94	2·42	+26·4 %	2·74	2·52	+20·9 %
	12	3·24	3·08	—	2·56	2·24	—	3·08	2·92	—
	14	3·18	2·62	+37·8 %	2·95	1·26	+11·1 %	2·90	2·62	+ 5·0 %
	16	3·14	2·30	—	2·82	0·80	—	2·83	1·07	—
	18	2·90	2·09	+26·3 %	2·78	0·10	+21·0 %	2·74	0·26	+10·9 %

The hypophysectomised rat is exceedingly sensitive to the thyreotropic hormone. It is seen here that one unit of the hormone twice a day causes a significant rise in the creatine excretion.

* Normal for hypophysectomised rats.

Since fairly small amounts of the thyreotropic hormone will produce a significant increase in creatine excretion in the normal rat, this may give us a satisfactory method of assaying the hormone. The simplicity of the test would

be a distinct advantage over our present method of measuring the increase in metabolic rate in the hypophysectomised rat.

Purified extracts of the growth hormone and of the adrenotropic hormone have been injected into normal rats and the creatine and creatinine excretion followed over a period of 18 days. The effect of thyreotropic extract which had been inactivated by boiling for 2 hours at p_H 5 was also studied. The average daily output of creatine on the eighth day of the growth extract was 0·71 mg. per 100 g., of the adrenotropic 0·55 mg., and of the boiled thyreotropic 0·38 mg., while the controls receiving active thyreotropic hormone excreted 2·88 mg. of creatine on the eighth day of injection.

A substance which inhibits the action of thyreotropic hormone in raising the metabolic rate has been reported by Collip and Anderson [1934; Anderson and Collip, 1934]. Creatine and creatinine excretion has been studied on a group of normal rats receiving the antithyreotropic extract, together with the thyreotropic hormone. The results are given in Table IV. A fall in metabolic rate

Table IV. *The effect of antithyreotropic and thyreotropic hormones upon the creatinine and creatine excretion of normal rats.*

		Group I (average of 4 rats)			Group II (average of 4 rats)		
	Day	Creatinine mg.	Creatine mg.	Metabolic rate	Creatinine mg.	Creatine mg.	Metabolic rate
Control period	2	2·81	0·72	—	2·92	0·60	Normal
	4	2·94	0·51	—	2·88	0·57	—
	6	3·00	0·82	—	2·86	0·50	—
	8	2·74	0·59	—	3·02	0·48	—
	10	2·86	0·68	Normal	2·90	0·60	Normal
Injections started on 10th day		Dose: thyreotropic 25 units b.i.d.			Dose: thyreotropic 25 units b.i.d.; antithyreotropic extract 1 ml. b.i.d.		
Experimental period	12	2·50	0·82	—	2·98	0·81	—
	14	2·95	1·05	+16·7 %	3·01	1·54	—
	16	2·90	1·22	—	3·12	1·21	−11·6 %
	18	3·10	1·74	—	3·14	1·15	—
	20	2·74	1·05	+37·4 %	2·58	1·07	− 6·0 %
	22	2·67	1·06	—	3·10	1·12	—
	24	2·70	0·82	—	3·00	0·89	—
	26	2·88	0·42	+20·1 %	3·18	0·66	−12·9 %
	28	2·66	0·76	—	2·88	0·51	—
	30	2·64	0·40	—	3·24	0·51	−20·1 %
	32	3·10	0·30	—	3·18	0·57	—
	34	2·80	0·20	+ 2·0 %	3·12	0·53	—

occurred in the group of animals receiving the antithyreotropic substance together with the thyreotropic hormone (Group II); however, the creatine excretion in this group was practically the same as in the group receiving thyreotropic hormone only (Group I).

SUMMARY.

1. The feeding of desiccated thyroid to normal rats caused a rise in the creatine excretion, which reached its peak on the tenth day and then gradually decreased while the metabolic rate was still rising.

2. The thyreotropic hormone caused a temporary increase in creatine excretion both in the normal and the hypophysectomised rat.

3. A significant increase in creatine excretion was produced with 6 units of thyreotropic hormone in the normal rat and with one unit in the hypophysectomised rat.

4. The thyreotropic hormone caused an increase in the creatine excretion of the dog.

5. Purified extracts of the growth hormone and of the adrenotropic hormone had no effect on the creatine excretion of normal rats. Thyreotropic extract inactivated by boiling also gave negative results.

6. Antithyreotropic substance, which inhibited the rise in metabolic rate by thyreotropic hormone, did not prevent an increase in creatine excretion.

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CLIII. THE INFLUENCE OF VITAMIN C (ASCORBIC ACID) ON PLANT AND ANIMAL AMYLASES.

By ARNULF PURR.

From the Cancer Research Laboratories, Graduate School of Medicine, University of Pennsylvania, Philadelphia, Pa.

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In a recent publication from this laboratory Purr [1933] reported on the effect of ascorbic acid on the action of the proteolytic enzymes. The present paper deals with the influence of ascorbic acid on plant and animal amylases which are important in cancer research on account of their relation to carbohydrate metabolism.

Kuhn [1925], in a fundamental work, studied the mutarotation of the products formed in the first stages of starch hydrolysis by amylases. He found that one group of amylases yields primarily α-maltose, which mutarotates downward (α-amylase), while another group yields β-maltose, which mutarotates upward (β-amylase). The amylases in pancreas and saliva belong to the α- type, while those of sprouted and unsprouted grains are of the β- type. These findings were later confirmed by Reichel [1932]. By studying the relation between the iodine reaction and the mutarotation of the starch decomposition products, it was found that the two types of amylase differ in their mode of action on the starch complex from the first point of contact. Thus, α-amylases are believed to break down first those groups in the starch complex which are responsible for the blue colour with iodine. Characteristically, the blue colour disappears with very low saccharification. On the other hand, β-amylases break down the starch in such a way that saccharification is almost complete while the blue colour remains unaltered. Through the discovery of this relation between saccharification and coloration with iodine, a simple method became available for distinguishing the amylases found in nature. It is merely necessary to allow the hydrolysis to proceed until a very definite colour tone, preferably the change from ultramarine blue to blue-violet, is reached and to determine the saccharification at that point. This is known as the transition point, and it expresses the amount of saccharification (in percentage maltose formed) at the point of colour change.

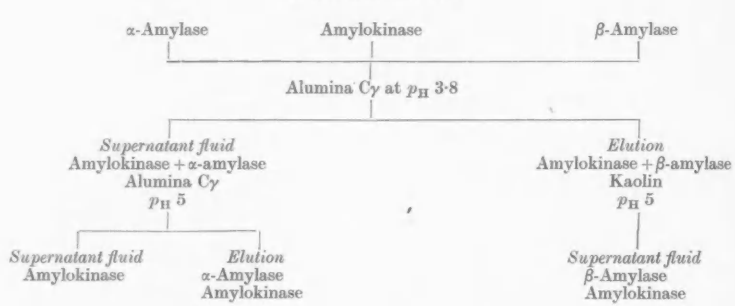
The transition points for several different types of amylase are as follows:

Transition point (percentage maltose formed).

Plant amylases		Animal amylases		
Barley	Green malt	Pancreas	Liver	Saliva
102 %	84 %	20 %	15 %	11 %
β-Amylase		α-Amylase		
Mutarotation upward		Mutarotation downward		
High saccharification		Low saccharification		
Iodine colour, blue-violet		Iodine colour, blue-violet		

By means of adsorption methods, Waldschmidt-Leitz, Reichel and Purr [1932] were able to isolate two amylases, α and β, from barley and green malt. While both amylases extracted from green malt were fully active, in barley the α-form was totally inactive and the β-form partially active. The difference between the barley enzyme system and the malt system lies in the formation of an activator of organic nature, "amylokinase," which arises during the process of sprouting.

The following scheme represents the process of separating the amylases of green malt.



This method, when applied to animal amylase systems, was unfortunately not so successful. However, the transition point of pancreas amylase indicates the presence of an animal β-type. As a result of the discovery, reported in this paper, of the specific activating effect of vitamin C on animal β-amylase, it has become possible to demonstrate the existence in pancreas of a β-type of amylase.

Vitamin C and animal amylases.

The effect of vitamin C on the activity of various animal amylases was investigated, using as source of enzyme the following: human saliva (0·2 ml. with 0·015 amylase units), acetone-ether-dried pig pancreas (0·1 ml. of a 1 : 10 aqueous extract with 0·035 amylase units), and rat-liver (1·5 ml. of a 1 : 5 aqueous extract with 0·0014 amylase units). To separate portions of the designated quantities of each of these were added (*a*) 8 mg. of ascorbic acid in 1 ml. of H_2O, (*b*) 8 mg. oxidised ascorbic acid in 1 ml. of H_2O, or (*c*) 1 ml. of H_2O. These mixtures were allowed to stand at 0° and p_H 7 for 5 minutes, after which 50 ml. of the stock solution were added. This contained 250 mg. of soluble starch (Lintner), 10 ml. of $M/15$ phosphate buffer of p_H 6·8 (or 10 ml. of $M/10$ citrate buffer of p_H 5·1), and 2·0 ml. of $M/5$ NaCl solution, in a total volume of 50 ml. The mixtures were incubated at 37° for the indicated periods of time, after which the enzyme action was stopped by addition of 2 ml. of N HCl. The amount of saccharification was determined by the method of Willstätter and Schudel [1918], the results being expressed in percentage maltose formed. The oxidised ascorbic acid was prepared by bubbling oxygen for 4 hours through a solution of 8 mg. of ascorbic acid in 1 ml. of H_2O at p_H 7. A trace of $FeSO_4,7H_2O$ was added to hasten the oxidation, the completion of which was determined by iodine titration.

Table I. *Ascorbic acid and animal amylases. Effect of* p_H.

Additions	p_H	Saliva		Pancreas		Liver	
		Time mins.	Maltose %	Time mins.	Maltose %	Time mins.	Maltose %
Water	6·8	10	22·6	8	37·04	60	13·72
Ascorbic acid	6·8	10	21·95	8	48·00	60	24·00
Oxidised ascorbic acid	6·8	10	22·00	8	46·64	—	—
Water	5·1	20	15·5	25	20·58	—	—
Ascorbic acid	5·1	20	15·9	25	19·20	—	—

From the figures obtained it can be seen that of the animal amylases tested, only the salivary amylase remained inactivated at both p_H 6·8 and 5·1. Pancreas and liver amylases, on the other hand, undergo considerable activation, but only at the optimum p_H for the enzyme reaction (6·8). In this respect the activation differs from that obtained with calcium salts, which occurs only at p_H 5·1, but not at 6·8.

The salivary amylase, as has been mentioned, has the lowest transition point found as yet, and therefore represents the purest α-type. The higher transition points of pancreas and liver amylases indicate the presence of mixtures of both α- and β-types. It was of interest to determine, therefore, whether these facts could be correlated with the non-activation of salivary amylase and the activation of pancreas and liver amylases by ascorbic acid. The investigations carried out in this direction, as shown in Table II, indicate that the activations obtained with ascorbic acid are related to a specific activation of the animal β-amylase.

Table II. *The effect of ascorbic acid on the transition point of pancreatic amylase.*

	Without ascorbic acid			With ascorbic acid		
	Saccharification			Saccharification		
Time mins.	Maltose mg.	Maltose %	Iodine colour	Iodine colour	Maltose mg.	Maltose %	
0	0·0	0·0	Ultramarine-blue	Ultramarine-blue	0·0	0·0	
4	20·0	8·0	,,	,,	37·5	15·0	
Transition point 10	45·0	18·0	Blue-violet	Blue-violet	70·0	28·0	Transition point
15	63·8	25·5	Violet	Violet	86·3	34·5	
20	80·0	32·0	Violet-red	Violet-red	102·5	41·0	
25	95·0	38·0	Red-violet	Red-violet	120·0	48·0	
30	112·5	45·0	Red-brown	Red-brown	136·3	54·5	

A large volume of a reaction mixture was prepared, containing 250 mg. of soluble starch, 10 ml. of $M/15$ phosphate buffer of p_H 6·8, 2 ml. of $M/5$ NaCl solution, and 1 ml. of a 1 : 10 aqueous extract of pancreas containing 0·015 amylase units (or the same amount of enzyme previously activated by 8 mg. of ascorbic acid), in a total volume of 50 ml. The mixture was incubated at 37°. The course of the reaction towards the transition point was followed by the method to be described later. At definite intervals, 50 ml. portions of the reaction mixture were removed and treated with 2 ml. of N HCl, and the amount of maltose formed was determined.

As shown in Table II, the activating effect of ascorbic acid is linked with an increase in the transition point. This indicates that ascorbic acid has a specific

activating effect on the β-amylase, and that the latter is present in pancreas and liver in an almost inactive state naturally.

Waldschmidt-Leitz and Purr [1932] were able to report an activating effect on animal amylase by calcium salts. As previously mentioned, this form of activation is sharply differentiated from that obtained with ascorbic acid in the conditions of p_H necessary. The same conclusion is reached by a study of the transition point, as shown in Table III.

Table III. *Activation of pancreatic amylase by $CaCl_2$ at p_H 5·1. Transition point.*

	Without $CaCl_2$			With $CaCl_2$			
	Saccharification			Saccharification			
	Time mins.	Maltose mg.	Maltose %	Iodine colour	Iodine colour	Maltose mg.	Maltose %	
	0	0·0	0·0	Ultramarine-blue	Ultramarine-blue	0·0	0·0	
	10	6·3	2·50	,,	,,	30·00	12·00	
	15	18·75	7·50	,,	Blue-violet	47·00	18·80	Transition point
	20	30·0	12·0	,,	Violet	62·50	25·00	
Transition point	25	47·5	19·0	Blue-violet	Violet-red	82·5	33·00	
	35	65·0	26·0	Violet	Red-violet	100·0	40·0	

Additions and procedure were the same as those for Table II, except that 10 ml. of $M/10$ citrate buffer of p_H 5·1 were used instead of the phosphate and 12 mg. of $CaCl_2$ instead of the ascorbic acid.

Calcium salts do not change the transition point, but only hasten its appearance, while ascorbic acid increases it. It is evident from these results that the effect of calcium salts is fundamentally different from that of ascorbic acid.

Procedure for the determination of the transition point.

Each of a series of test-tubes contained 2 ml. of distilled water and 3 drops of $N/100$ iodine solution. At definite time intervals, 1 ml. of the enzyme reaction mixture was added, until the first definite blue-violet colour could be recognised. Immediately, 50 ml. of the hydrolysing mixture were removed, 2 ml. of N HCl were added to stop the enzyme reaction, and the sugar formed was estimated by the Willstätter-Schudel method.

It should be mentioned that the method can be used for quantitative comparisons only when no substances are present which take up iodine in neutral or acid solution. When these conditions exist, the method is in all cases inapplicable; for the presence of large quantities of such reducing substances (cell residue, tissue suspensions, thiosulphate, *etc.*), leads to the formation of hydrogen iodide or its salts. The latter compounds, as is known, weaken the colour of the starch-iodine complex, and therefore interfere with the colour comparison. These substances affect the starch-iodine colour in a manner similar to that of amylase itself and may easily lead to erroneous conclusions.

In order to eliminate this source of error when using ascorbic acid, which takes up iodine very readily, it is necessary, in the determination of the transition point, to compensate for its presence. The method of procedure was the same as previously described, except that to those test-tubes in which the

transition point of pancreatic amylase alone was to be determined, 0·16 mg. of ascorbic acid was previously added. This amount corresponds to that present in 1 ml. of the enzyme mixture which is being activated by ascorbic acid. In each case, then, equivalent amounts of ascorbic acid were present, so that the error due to formation of hydrogen iodide was eliminated. Because of the consumption of iodine which occurs in the presence of ascorbic acid, it was necessary to add 10 instead of 3 drops of $N/100$ iodine to each test-tube, in order to have the necessary excess for the determination and for stopping the enzyme reaction.

The next series of experiments (Table IV) shows the effect of several amino-acids and of insulin on the activity of pancreatic amylase at p_H 6·8 and 5·1. The procedure was exactly the same as that described in Table I, except for the substitution of the indicated amounts of amino-acids and insulin for the ascorbic acid.

Table IV. *The effect of amino-acids on the activity of pancreatic amylase.*

	p_H 6·8			p_H 5·1		
Additions	Time mins.	Saccharification Maltose %	Activation Maltose %	Time mins.	Saccharification Maltose %	Activation Maltose %
None	7	14·7	0	20	12·00	0
Cysteine (20 mg.)	7	14·9	0	20	18·00	6·0
Cystine (20 mg.)	7	14·7	0	20	21·50	9·50
Alanine (20 mg.)	7	14·7	0	20	12·00	0
Histidine monohydrochloride (20 mg.)	7	14·8	0	20	23·00	11·0
Proline (20 mg.)	7	14·5	0	20	12·00	0
Insulin—132 units (9·5 mg.)	7	14·8	0	20	22·00	10·00

It is clear from this table that the p_H of the amino-acid activation agrees with that observed in the case of calcium activation. The agreement also can be seen in a study of the transition points (these figures are not presented in this paper). This activation therefore also differs sharply from that obtained with ascorbic acid.

The extent to which the activating influence of ascorbic acid is exerted on the complicated mechanism of glycolysis will be reported in a later communication.

Vitamin C and plant amylases.

The significance of ascorbic acid as an enzyme regulator is not limited to the amylases of animal origin, but as the following experiments show, it must also be credited with a similar function with regard to plant amylolytic processes.

The plant amylases investigated were the following: barley (0·5 ml. of a 1 : 5 aqueous extract, with 0·030 amylase unit), green malt (0·25 ml. of a 1 : 5 aqueous extract with 0·030 amylase unit), barley amylase purified by alumina Cγ, β-form (1·0 ml. with 0·015 amylase unit), and the green malt amylase purified by alumina Cγ, α-form (3·0 ml. with 0·015 amylase unit). To the designated amounts of each of these were added (*a*) 8 mg. of ascorbic acid in 1 ml. of H_2O, (*b*) 8 mg. of oxidised ascorbic acid in 1 ml. of H_2O or (*c*) 1 ml. of H_2O. The mixtures were allowed to stand 10 minutes at 37°, after which 50 ml. of the stock solution of p_H 5·1 previously described were added. The incubation and sugar determinations were carried out exactly as previously described. The purified β-amylase from barley was prepared by treating 10 ml. of 1 : 5 barley extract containing 1·5 amylase units with alumina Cγ (8 ml. = 60 mg. Al_2O_3) at

p_H 3·8 (2 ml. of *M* acetate buffer). The adsorbate was eluted with 10 ml. of *M*/15 secondary phosphate and brought to p_H 7 by the addition of *N*/10 HCl. The purified α-amylase was prepared by adsorbing 10 ml. of 1 : 5 green malt extract eight times with alumina Cγ (145 mg. × 8 of Al_2O_3) at p_H 3·8 (2 ml. of *M* acetate buffer). Compare the schema, p. 1142.

Table V. *Effect of ascorbic acid on plant amylases.*

Enzyme	No additions		Ascorbic acid		Oxidised ascorbic acid	
	Time mins.	Maltose %	Time mins.	Maltose %	Time mins.	Maltose %
Barley (unpurified)	9	35·68	7	12·34	7	36·36
Malt (unpurified)	10	37·04	10	19·90	10	28·81
β-Amylase (purified from barley)	20	37·70	20	10·37	10	36·36
α-Amylase (purified from malt)	9	19·90	10	16·46	10	11·90

While the animal amylases are either activated or else not affected at all by ascorbic acid, the plant amylases thus far investigated are inhibited. This inhibition is dependent not only on whether reduced or oxidised ascorbic acid is used, but also on the type of amylase, α or β. As shown in Table V, the β-type of plant amylase is very strongly inhibited by reduced ascorbic acid, but is unaffected by the oxidised form. This behaviour is shown both by the purified enzyme, and by the crude green malt extract which contains amylokinase. The opposite picture appears in the case of plant α-amylase, whose activity, as has been previously mentioned, is dependent on the presence of amylokinase. The activity of this form is inhibited only by the oxidised form of ascorbic acid, while it is scarcely affected by the reduced form. The slight inhibition which may be observed can be explained by the presence of a small amount of β-amylase which remains after the purification.

These findings extend the knowledge of the essential differences between the plant and animal amylases which are summarised in Table VI.

Table VI. *Comparative behaviour of plant and animal amylases toward various reagents.*

+ = Activation; − = Inhibition; 0 = Indifferent.

Reagent	p_H	Plant amylases		Animal amylases	
		β	α	β	α
Amylokinase	5·1	+	+	0	0
,,	6·8	+	+	0	0
Vitamin C	5·1	−	0	0	0
,,	6·8	−	0	+	0
Oxidised vitamin C	5·1	0	−	0	0
,,	6·8	0	−	+	0
NaCl	5·1	0	0	0	+
,,	6·8	0	0	0	+
$CaCl_2$	5·1	0	0	0	+
,,	6·8	0	0	0	0
p_H optimum		5·1	5·1	6·8	6·8

Table VII shows the effect of oxygen on the plant amylase complex from barley in the presence and absence of ascorbic acid. Oxygen was bubbled through a barley amylase extract for 4 hours in the presence and absence of ascorbic

acid. A trace of $FeSO_4$ was added as an oxidation catalyst. The determinations of amylase activity after the oxygenation were carried out exactly as previously described. The results obtained indicate that ascorbic acid may also exert an effect in protecting the amylase from oxidation.

Table VII. *Effect of oxygen on barley amylase in the presence and absence of ascorbic acid.*

	Barley amylase without vitamin Maltose (%)	Barley amylase with 8 mg. vitamin Maltose (%)
4 hour treatment with oxygen	12·3	20·6

Recently Virtanen *et al.* [1933] found a relation between growth of plants and vitamin C content. In view of the connection between vitamin C and the amylases reported in the present paper, it is of interest to present the results of an investigation[1] of the changes occurring in the activities of the several kinds of amylases during the ripening of various grains.

The crushed grains at different stages of ripening were extracted for 24 hours with 5 parts of water, toluene being added as antiseptic, and then filtered by suction. The reaction mixtures consisted of 140 mg. of amylo-amylase (prepared according to Samec), 10 ml. of citrate buffer of p_H 5·1, and aliquot parts of the grain extracts containing 0·01 amylase unit, in a total volume of 50 ml. The mixtures were incubated at 37°, and the course of the reaction towards the transition point was followed as previously described. The mutarotation was determined by the method of Kuhn [1925]. A 2 dm. polarimeter-tube was filled with the reaction mixture, and the rotatory power of the solution was determined at intervals, first without carbonate, and again after ½ hour standing with 0·5 g. of anhydrous sodium carbonate. The difference between the two determinations expresses the mutarotation, the magnitude of which is dependent on the experimental procedure. The decisive point is the direction of the mutarotation (− for α-; + for β-amylase).

Table VIII. *Relation between amylase activity and the ripening process of various grains.*

Grain	State of grain content	Transition point Maltose %	Mutarotation at transition point	Remarks
Barley	Milky fluid	77·5	+0·07°	For comparison, green malt has a mutarotation of +0·15 at the transition point (84 % maltose)
	Soft mass	67·3	+0·06	
	Mealy mass	82·1	+0·08	
	Ripe grain	96·1	+0·10	
Rye	Milky fluid	88·2	+0·13	
	Soft mass	68·0	+0·08	
	Mealy mass	76·0	+0·09	
	Ripe grain	83·3	+0·05	
Oats	Milky fluid	80·85	+0·15	
	Soft mass	60·7	+0·08	
	Mealy mass	75·0	+0·06	
	Ripe grain	90·0	+0·10	

On the basis of these results, it can be seen that in the first stages of the development of grains, the relationships between the different amylase types

[1] This investigation was carried out at the Biochemical Institute of the Deutsche Technische Hochschule, Prague. The various kinds of grains used in each stage of the ripening process were obtained from J. Purr, Grabschuetz, Czechoslovakia.

(α or β) are similar to those existing in sprouting grains such as green malt. The nearer the grain approaches to ripening, the higher is the transition point, until it finally reaches the value for fully ripened, dormant grain. The increase in the transition point indicates that the α-amylase is gradually becoming inactive, and from this it may be concluded that the α-enzyme in growing (including sprouting) plant cells is involved as an energy-supplying factor in the enzymic intermediate processes. It is probable that the decrease in vitamin C during the ripening of grains, as found by Virtanen *et al.* [1933], is related to the inactivation of the α-amylase. This working hypothesis is supported by the finding that oxidised vitamin C inhibits plant α-amylase. Whether amylokinase arises during the ripening process, and its relation to vitamin C, must be determined by further experiment.

Summary.

1. It has been shown that vitamin C (ascorbic acid) is a specific activator for the β-type of animal amylase. β-Amylase occurs in animal organs in a practically inactive state.

2. Vitamin C exerts an inhibiting effect on the β-type of plant amylase but has no effect on the α-type. The oxidised form of ascorbic acid has no effect on the β-form but inhibits α-amylase of plants.

3. A study of the relation between amylase activity and the growth of grains leads to the conclusion that in the first stages of growth the same amylase relationships exist as in the sprouting grain. The fact that the α-components become inactive at the end of the growth period is in agreement with the findings of Virtanen on the vitamin C changes which occur, and with the fact that the oxidised form of the latter inhibits α-plant amylase.

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